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# COMPARATIVE GENOMICS, ANTIMICROBIAL RESISTANCE DETERMINANTS, AND PATHOGENICITY OF COMMUNITY-ASSOCIATED *STAPHYLOCOCCUS AUREUS*

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**by**

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## Dedication

This dissertation is dedicated to my husband, Kevin, whose sacrificial love, support, humor, and encouragement have inspired me to pursue and complete this research.

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I wish to express my sincere thanks to my dissertation supervisor, Dr. Christopher Frei, for his guidance, encouragement, and support throughout my research journey. Thank you for believing in me to pursue and complete this dissertation research. In addition, I express my appreciation to my dissertation committee members, Dr. Yufeng Wang, Dr. Randall Olsen, Dr. Kenneth Lawson, and Dr. Jim Wilson. Thank you for your valuable guidance, support, and patience throughout this dissertation project. I would also like to acknowledge all who, directly or indirectly, have lent their helping hand in this venture.

# COMPARATIVE GENOMICS, ANTIMICROBIAL RESISTANCE DETERMINANTS, AND PATHOGENICITY OF COMMUNITY-ASSOCIATED *STAPHYLOCOCCUS AUREUS*

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The University of Texas at Austin, 2016

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*Staphylococcus aureus* is a major human pathogen and a global public health issue. It is considered an opportunistic pathogen as it asymptomatically colonizes its host, but can occasionally cause diseases that range in severity from relatively minor skin and soft tissue infections (SSTI) to life-threatening cases of pneumonia and endocarditis. There is a critical need to better understand mechanisms that lead to the evolution, resistance, and severity of *S. aureus* infections. Bacterial whole genome sequencing (WGS) techniques have offered new insights into *S. aureus* genomic populations and have the potential to predict antimicrobial resistance and infection severity. This study applied WGS 1) to describe the diversity and distribution of resistance mechanisms among community-associated *S. aureus* isolates, and 2) to identify *S. aureus* genetic signatures associated with SSTI isolates and derive a predictive risk model. WGS was performed on *S. aureus* isolates from patients within 14 primary care clinics in the South Texas Ambulatory Research Network from 2007 to 2015. The bacterial genomes were compared to a reference genome, FPR3757 (USA300

strain) to identify single nucleotide polymorphisms (SNPs). Phylogenetic analyses were conducted using concatenated SNP nucleotides in the core genomes. In the first study, the resistome was assembled by identifying antimicrobial resistance determinants related to the phenotypically derived antibiogram. The findings of this study identified that multidrug-resistant *S. aureus* isolates have emerged in the South Texas community; approximately one-third were multidrug-resistant. There was an increasing resistance pattern to fluoroquinolones. Furthermore, the genotype demonstrated to be highly predictive of antimicrobial resistance (very major error rate=0% and major error rate=1.4%). These findings highlight the genomic diversity of *S. aureus* strains in the South Texas community and demonstrate the utility of next generation sequencing to define the diversity and distribution of resistance mechanisms within *S. aureus.* Further work to explore antimicrobial selective pressures is needed. The second study utilized a bacterial genome-wide association study to identify specific variants associated with *S. aureus* pathogenicity. This study revealed the heterogeneity of *S. aureus* SSTI and nasal colonization isolates and identified potentially novel pathogenic mechanisms.

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# CHAPTER ONE

## *Staphylococcus aureus* infection epidemiology

*Introduction to Staphylococcus aureus infections*

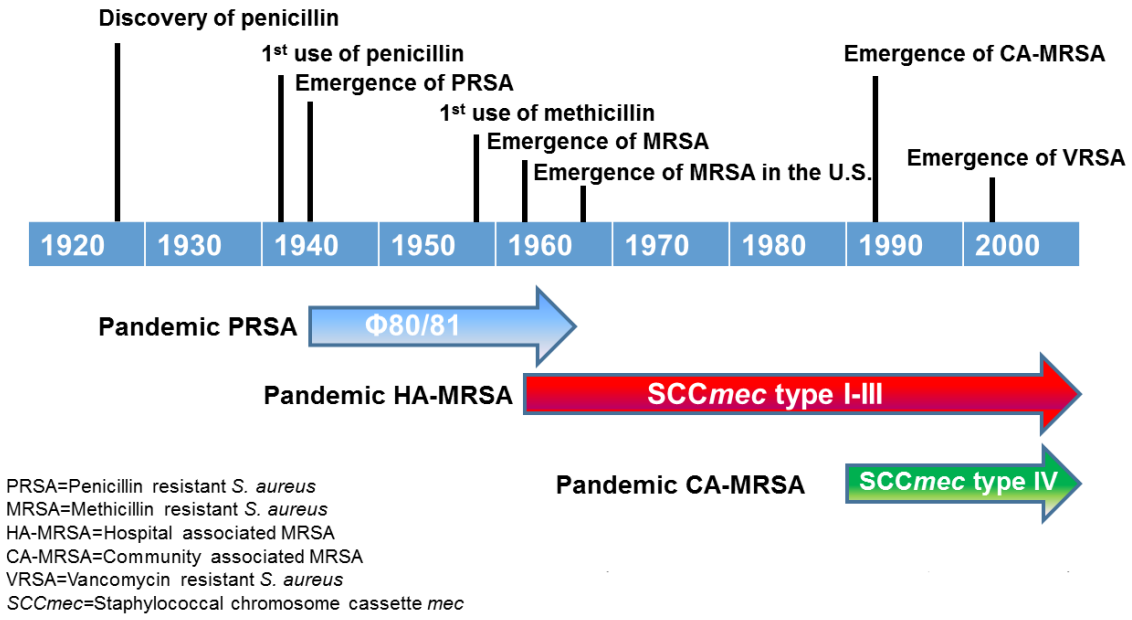
*Staphylococcus aureus* is a Gram-positive cocci bacterium known to cause significant morbidity and mortality. It typically exists as a commensal bacterium, colonizing approximately one-third of the U.S. population. *S. aureus* is an opportunistic pathogen, having the ability to cause a wide range of infectious syndromes in humans, from relatively minor skin infections to more severe life-threatening infections such as sepsis and endocarditis. It is a highly adaptive bacterium capable of producing a wide array of virulence factors and resistance mechanisms. These features allow *S. aureus* to evade host defense mechanisms, cause rapid dissemination, and acquire antimicrobial resistance. *S. aureus* represents a major threat to the American public. There has never been a more crucial time to develop interventions to prevent and effectively treat *S. aureus* infections.

*Evolution of S. aureus*

*Staphylococcus* was first discovered in 1880 by the surgeon Sir Alexander Ogston in Aberdeen, Scotland. He observed grape-like clusters of bacteria under the microscope, which was therefore named *Staphylococcus* from the Greek expression “a bunch of grapes”. A few years later, Friedrich Rosenbach gave its formal nomenclature, *Staphylococcus aureus* for its characteristic “gold” pigmented appearance of colonies.[1]

Since its discovery, *S. aureus* has demonstrated a remarkable ability to evolve and acquire resistance to almost any antibiotic. Infections caused by antibiotic resistant *S. aureus* have reached epidemic proportions globally. Beginning in the 1940s, several “waves of resistance” by *S. aureus* have occurred (Figure 1.1).[2] The first wave was described after the initial use of the first known antibiotic, penicillin, with the emergence of penicillinase-producing *S. aureus* infections. These infections were primarily caused by a *S. aureus* clone known as phage-type 80/81.[3-5] After the introduction of methicillin, the pandemic phage-type 80/81 largely declined. However, the prevalence of different penicillinase-producing *S. aureus* strains continues to remain high.[6] The second wave of resistance began shortly after the use of methicillin. In 1961, the first case of methicillin-resistant *S. aureus* (MRSA) was reported followed by the first MRSA outbreak in 1963.[7]

**Figure 1.1** Evolution of *S aureus*




For decades, MRSA infections were limited to hospitals, and affected mostly older patients with comorbid conditions and those exposed to the health care setting. However, in the last three decades, there has been a dramatic rise of MRSA infections in the community setting, known as community-associated methicillin-resistant *S. aureus* (CA-MRSA). These CA-MRSA strains have rapidly disseminated and are endemic in most areas of the U.S. In contrast to health care-associated MRSA (HA-MRSA), CA-MRSA strains most commonly affect healthy individuals without traditional risk factors or health care exposures.[8-10] A timeline of the emergence of CA-MRSA infections is depicted in Table 1.1. Among these

reports, the most common manifestation of CA-MRSA is skin and soft tissue infections (SSTIs).

**Table 1.1** Timeline of the emergence of CA-MRSA infections[11-18]

| Years | Report description |
|---|---|
| 1980-1981 | First report of CA-MRSA in Detroit, MI |
| 1989-1998 | Outbreaks in geographically separate regions; Australia, Papua New Guinea, Turkey, New Zealand |
| 1993-1994 | Outbreak in a high school wrestling team in Vermont |
| 1997 | Outbreak in Native American population in Midwest U.S. |
| 1997-1999 | 4 pediatric deaths due to septic shock caused by CA-MRSA |
| 1997-2003 | Outbreaks in prison populations in Los Angeles and San Francisco |
| 1998 | Outbreak in a rugby team in Great Britain |
| 2002-2003 | Outbreaks reported in HIV+ people and men who have sex with men |
| 2003 | Outbreak among athletes of the St. Louis Rams professional football team |

CA-MRSA=community-associated methicillin resistant *S. aureus;* HIV=human immunodeficiency virus

*Classification of S. aureus*

Classification of *S. aureus,* particularly MRSA, has traditionally been classified into HA-MRSA and CA-MRSA. However, the HA-MRSA and CA-MRSA classifications are no longer distinct.[19-21] Recent surveillance has shown that HA-MRSA can spread to community contacts and CA-MRSA is becoming a significant cause of health care associated.[22-26] Several epidemiological criteria have been used to classify MRSA infections, although the classification scheme has not been standardized.[27-29] The general criteria for classification include the following: the time of isolation, host risk factor profiles, antimicrobial susceptibility patterns, and molecular characteristics of the isolate (Table 1.2).

**Table 1.2**[24,29,30] Classification of MRSA

| Classification | Definition |
|---|---|
| HA-MRSA | MRSA infection in a patient with one of the following risk factors: presence of an invasive device at time of infection, history of HA-MRSA, history of surgery, hospitalization, dialysis, or residence in a long-term care facility in the 12 months preceding culture. |
| CA-MRSA | MRSA infection with onset in the community in a patient who is without risk factors for HA-MRSA. |
| Hospital-onset | The CDC ABC Surveillance System defines invasive hospital-onset HA-MRSA cases with positive culture result from a normally sterile site obtained >48 hours after hospital admission. |
| Community-onset | The CDC ABC Surveillance System defines invasive community-onset HA-MRSA as cases with onset in the community and at least one of the HA-MRSA risk factors. |

HA-MRSA=health care associated methicillin resistant *S. aureus*; CA-MRSA=community-associated methicillin resistant *S. aureus*; CDC=the US Centers for Disease Control and Prevention; ABC Surveillance System=the Active Bacterial Core Surveillance Program.

MRSA strains have been characterized by certain bacteriologic features including antimicrobial susceptibility patterns and genotype. Molecular subtyping

has been important in tracking the spread of infections and transmission of strains, determining source of infections, monitoring trends, and confirming the effectiveness of established prevention efforts. Numerous approaches have been used for subtyping of MRSA. Common approaches include the subtyping of the staphylococcal cassette chromosome element (SCCmec), pulsed-field gel electrophoresis (PFGE), carriage of Panton Valentine Leukocidin (PVL) genes, and multilocus sequence typing (MLST).

PFGE is commonly used for molecular typing of *S. aureus* isolates in the US.[31-34] This method relies on separation of SmaI-digested *S. aureus* genomic DNA fragments according to size in an agarose gel by pulsed-field electrophoresis. Related strains are clustered according to an 80% similarity coefficient. The CDC developed a national PFGE database for *S. aureus* that uses the "USA" designation. PFGE types USA100, USA200, USA500, USA600, and USA800 are predominant health care–associated strains, whereas USA300 is the predominant community-associated *S. aureus* strain. The USA300 designation has been used to characterize ST8 and PVL-positive CA- MRSA.[21]

MRSA strains can be characterized by the mobile genetic element (MGE), SCCmec, which harbors the *mecA* gene encoding for methicillin resistance.[35] To date, there have been 11 SCCmec elements that have been identified. HA-MRSA typically carry a large SCCmec element belonging to type I, II, or III. SCCmec II (53 kb) and SCCmec III (67 kb) are large and possess other mobile genetic elements, such as integrated plasmids (e.g., pUB110, pI258, and pT181) and

transposons (e.g., Tn554).[36] In contrast, CA-MRSA strains carry smaller elements, SCCmec IV (21–24 kb) and V (27 kb), are generally susceptible to non-beta-lactam antibiotics, and frequently carry PVL genes.[2,37]

MLST is a genotyping method based on sequences. This method analyzes single nucleotide variations (e.g. alleles) within 7 housekeeping genes in *S. aureus*. Isolates that have identical sequences are considered a clone and are assigned a unique sequence type (ST). *S. aureus* isolates with similar identity at 5 of the 7 housekeeping genes based on MLST are grouped into major lineages, named clonal complexes (CC).[37,38] CCs can be constructed based on ST's through a web-implemented algorithm called BURST (Based Upon Related Sequence Types), allowing the expansion or emergence of clones to be put in the context of their genetic background. MRSA strains have been generally grouped into a subset of CCs, including CC1, CC5, CC8, CC22, CC30, and CC45.[39,40,41]The first MRSA clinical isolates (COL as an example) were ST250, belonging to the CC8 lineage. The predominant CA-MRSA strains have been classified as ST8 belonging to the CC8 lineage.[39] MLST has been useful to identify outbreaks and expansions; however, different algorithms and more genomic information are required to determine more deep-rooted relationships within the population.

In summary, the molecular distinction between HA-MRSA and CA-MRSA is increasingly blurred. It has become more difficult to determine whether MRSA strains associated with outbreaks are related to health care workers, other patients, the hospital environment, or the community.[21,22,42-45] Rapid WGS has

proven to be much more discerning; but, has yet to be widely available. In general, HA-MRSA strains tend to have a multidrug-resistant profile, carry SCCmec type II or III, and have a USA100 or USA200 PFGE pattern. Comparatively, CA-MRSA strains are typically susceptible to non β-lactam antibiotics, carry SCCmec type IV or V, and typically have a USA300 or USA400 PFGE pattern. Table 1.3 provides a summary.

**Table 1.3** Major clonal complexes of *S. aureus* and select genome sequencing projects[46]

| Clonal Complex | Multilocus Sequence Type | Common Names and/or PFGE types | Comment | Whole genome Accession No |
|---|---|---|---|---|
| CC1 | ST1 | USA400 and MW2 | First US CA-MRSA strain, SCCmecIV and PVL-positive, pediatric fatal septicemia, USA, 1998 | BA000033 |
| CC5 | ST5 | USA100 and New York/Japan clone, | Most common US health care–associated MRSA, | BA000017 |
| | ST5 | Mu50 | SCCmecII | |
| | | USA800 and pediatric clone/EMRSA | Among earliest recognized SCCmecIV-bearing strains. | |
| CC8 | ST250 | COL | First (archaic) MRSA clinical isolate, SCCmecI, UK 1961 | CP000046 |
| | ST247/ST239 | Iberian Clone | Descendant of COL | |
| | ST239 | EMRSA-5 | SCCmecIII | |
| | ST254 | Brazilian, EMRSA-1 | SCCmecIV | |
| | ST8 | EMRSA-10 USA300, FPR3757, LAC | Predominant US CA-MRSA strain, SCCmecIV and PVL-positive, ACME-positive, | CP000255 |
| | ST8 | | Abscess, USA 2000 | CP000253 |
| | ST8 | | MSSA, UK, 1949 | AP009351 |
| | | NCTC8325 Newman | MSSA, clumping factor overproducer UK, 1941 | |
| CC22 | ST22 | EMRSA-15 | International clone, prominent in Europe and Australia | |
| CC30 | ST30 | USA1100 | Uncommon US CA-MRSA, SCCmecIV and PVL-positive | |
| | ST30 | SWP | Southwest Pacific clone, CA-MRSA, SCCmecIV and PVL-positive | |
| | ST36 | USA200 and EMRSA16 | Single most abundant cause of MRSA infections in UK; second most common cause of MRSA infections in US hospitals in 2003 | BX571856 |
| CC45 | ST45 | USA600 | SCCmecII | |

CC=clonal complex; PFGE=pulsed-field gel electrophoresis; ST=sequence type.

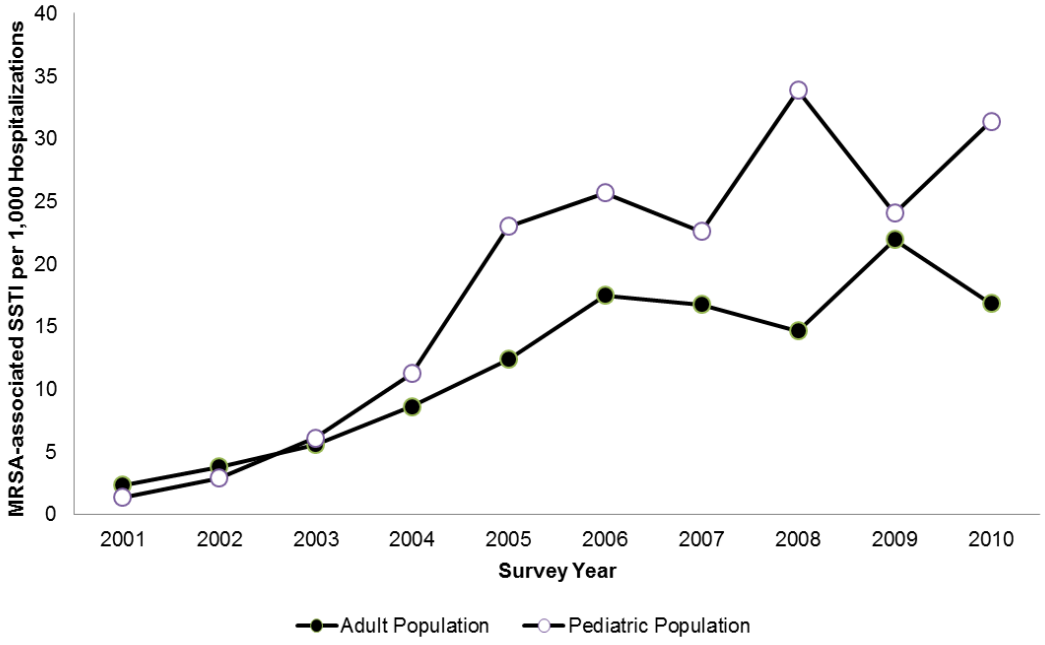
*Burden of illness due to S. aureus associated SSTIs*

*S. aureus* is a leading cause of infections globally, and is responsible for more annual deaths in the U.S. than HIV. In 2011, MRSA was responsible for 80,461 cases of invasive disease and 11,285 related deaths in the U.S.[30]

While *S. aureus* has traditionally been a leading cause of SSTIs, its prominence has dramatically increased in parallel with the emergence of CA-MRSA. A study based in Texas found a 7-fold increase in the incidence of CA-MRSA infections from 1997 to 2000 compared to 1990 to 1996.[47] By the mid-2000's, CA-MRSA accounted for more than 50% of skin abscesses in U.S. emergency departments and hospitals.[48] In the primary care setting, CA-MRSA accounts for 38% to 61% of culture-positive SSTIs.[49-51] In 1997 to 2005, the annual incidence of outpatient visits for SSTIs increased 50%, from 32 per 1000 to 48 per 1000 visits. *S. aureus* associated SSTI hospitalizations have also increased.[52] Klein et al. reported that the estimated number of MRSA-related hospitalizations across all infection types doubled from 127,036 in 1999 to 278,203 in 2005.[53] In a study using the Nationwide Inpatient Sample, Suaya et al. reported that *S. aureus* SSTI associated hospitalizations increased 123% between 2001 and 2009, with the highest increase in incidence seen among children 0-17 years of age. The investigators noted that the observed increase in *S. aureus* hospitalizations occurred simultaneously with the emergence of the CA-MRSA USA300 clone.[54] Similarly, Frei et al. found that pediatric hospitalizations for both *S. aureus* associated SSTIs increased from 1996 to 2006.[55] Recently, Lee et al. reported that

MRSA SSTI associated hospitalizations in the U.S. has increased more than 30-fold among pediatrics, and 8-fold among adults from 2001 to 2010 (Figure 1.2) (unpublished data).

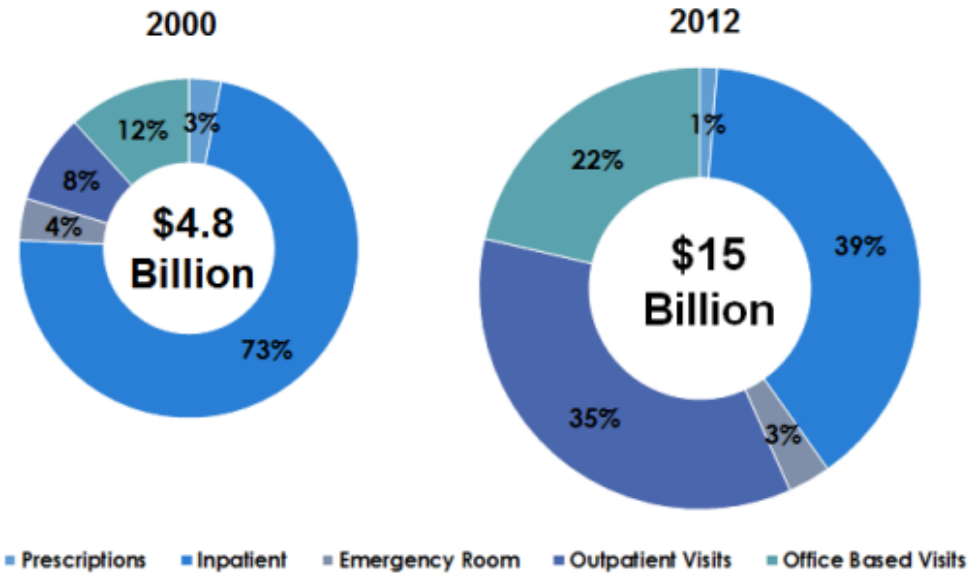
**Figure 1.2** Adult and pediatric hospitalizations for MRSA associated SSTIs in the United States from 2000 to 2010



*Cost of illness due to S. aureus SSTIs*

The costs associated with the management of *S. aureus* infections can be substantial. Costs vary by population, setting, cost perspective, and treatment approach. In U.S. hospitals, the treatment of *S. aureus* infections cost more than $14.5 billion annually.[54] In 1997, Marton et al. reported that the mean overall cost of an SSTI episode among inpatients and outpatients was $8,865.[56] In 2006, Menzin et al. estimated that the mean associated hospitalization cost for *S. aureus* SSTIs was $6,800.[57] In a study among hospitalized patients with gram-positive bacteria associated SSTIs, the median charge was $19,894.[58] Recently, Lee et al. estimated the total direct health care costs of SSTIs to be $14.9 billion in 2012, representing a 3-fold increase from $4.8 billion in 2000. This was largely driven by an 8-fold increase in ambulatory expenditures for SSTIs (Figure 1.3). The mean expenditures for ambulatory visits increased 5-fold from $253 [standard error (SE) $\pm$ $33] in 2001 to $1,336 (SE $\pm$ $240) in 2010 (unpublished data).

**Figure 1.3** Proportion of direct expenditures for SSTIs by health care service in the United States, 2000 and 2012



*Risk factors for S. aureus SSTIs*

CA-MRSA causes infections in many different hosts, ranging from young healthy individuals without traditional risk factors to those with extensive underlying diseases and health care exposures.[59] Prior studies have identified several risk factors for CA-MRSA SSTIs. Table 1.4 highlights common risk factors for CA-MRSA SSTIs. SSTIs due to MRSA predominantly affect children, adolescents, and middle-aged adults; the median age for adults ranges from 20 to 47 years. CA-MRSA SSTIs have been found to be more frequently identified among males, African Americans, and those with a history of a prior skin infection.[13,19,23,47,51,60-73] Crowding and sharing of personal items are also important factors.[60,66,68] The transmission of CA-MRSA has been demonstrated through direct contact among football players, wrestlers, and military trainees.[33,60,62] Household spread of CA-MRSA has proven to be an important reservoir for transmission.[74,75] In approximately 15% of cases, MRSA-infected patients reported having close contact with a person who had a similar skin infection. While prior antimicrobial exposures have been inconsistently associated with MRSA, the risk appears to be highest for fluoroquinolone and macrolide exposures. Furthermore, other host risk factors such as obesity and body size have been reported.[63,64,69,76]

Recently, Lee et al. performed a case-case-control study within 14 primary care clinics in South Texas from 2007 to 2015. Overall, 325 patients [*S. aureus* SSTI cases (Case Group 1)=175; MRSA SSTI cases (Case Group 2)=115; MSSA SSTI cases (Case Group 3)=60; uninfected control group (Control)=150 were

evaluated]. Each Case Group was compared to the control group, and then qualitatively contrasted to identify unique risk factors associated with *S. aureus,* MRSA, and MSSA SSTIs. Overall, prior SSTIs, male gender, and non-healthcare occupation were predictors for *S. aureus* SSTIs. The only unique risk factor for CA-MRSA SSTIs was a high body weight (publication in review).

**Table 1.4** Summary of clinical studies identifying risk factors for CA-MRSA SSTIs

| Study | Geographic area | Setting | Population | Comparator Group | Risk factors |
|---|---|---|---|---|---|
| Moran[48] (2007) n=422 | 11 U.S. cities | ED | SSTIs | Other pathogens | Antibiotic use in the past month, non-Hispanic African American, reported spider bite, history of MRSA infection, close contact with persons with skin infection |
| King[77] (2006) n=389 | Atlanta, GA | Outpatient, Hospital | *S. aureus* SSTIs | CA-MSSA | Female gender, African-American, hospitalization within the past year |
| Hota[67] (2007) n=1,222 | Chicago, IL | ED, Outpatient, Hospital | *S. aureus* | CA-MSSA | Younger age, HIV infection and incarceration within the past year, African American |
| Lee[71] (2005) n=111 | Los Angeles, CA | Outpatient | MSM, HIV | Control | Sex partner with skin infection, methamphetamine use, routine hand-on contact with customers at work, frequent fingernail biting, routine use of a public hot tub or sauna |
| Al-Rawahi[78] (2008) n=190 | Vancouver, Canada | ED | MRSA | HA-MRSA | Presence of abscesses or cellulitis, injection drug use |
| Campbell[62] (2004) n=202 | San Diego, CA | Outpatient | Military personnel | Control | Close contact with persons with skin infection, having family or friends working in health care setting |
| Kazakova[79] (2005) n=53 | St. Louis, MO | Outbreak | Football Players | Non-SSTI controls | Being the lineman or linebacker position, high BMI |
| Coronado[80] (2007) n=175 | New York, NY | Outbreak | Religious community | Non-SSTI controls | Antibiotic use within 12 months, sauna use |
| Khawcharoenporn[69] (2010) n=137 | Honolulu, HI | Outpatient | SSTI | SSTI due to other bacteria | Presence of abscesses, obesity (BMI $\geq$ 30) |

**Table 1.4** (Cont.) Summary of clinical studies identifying risk factors for CA-MRSA SSTIs

| Study | Geographic area | Setting | Population | Comparator Group | Risk factors |
|---|---|---|---|---|---|
| Casey[63] (2013) n=78,216 | U.S. | Hospital, Outpatient | SSTIs | Controls, HA-MRSA | Age, season, community socioeconomic deprivation, obesity, smoking, prior SSTI, and antibiotic administration |
| Landrum[81] (2012) | U.S. | Hospital | SSTIs | HA *S. aureus* | Age 18-24 years, men, active duty, living in the Southern U.S. |
| Cadena[61] (2015) n=112 | San Antonio, TX | Hospital, Outpatient | MRSA nasal carriers at discharge | Controls | Prior hospital admission, prior MRSA infection, myocardial infarction |
| Lee (2015) n=325 | South Texas | Outpatient | *S. aureus* SSTIs | CA-MSSA, Controls | Prior SSTI and body weight $\geq$ 110 kg |

CA-MRSA=community-associated methicillin-resistant *Staphylococcus aureus*; CA-MSSA=community-associated methicillin susceptible *Staphylococcus aureus*; ED=emergency department; HA-MRSA=healthcare-associated methicillin resistant *Staphylococcus aureus*; HIV=human immunodeficiency virus; MSM=men who have sex with men; SSTI=skin and soft tissue infection

*Treatment of S. aureus SSTIs*

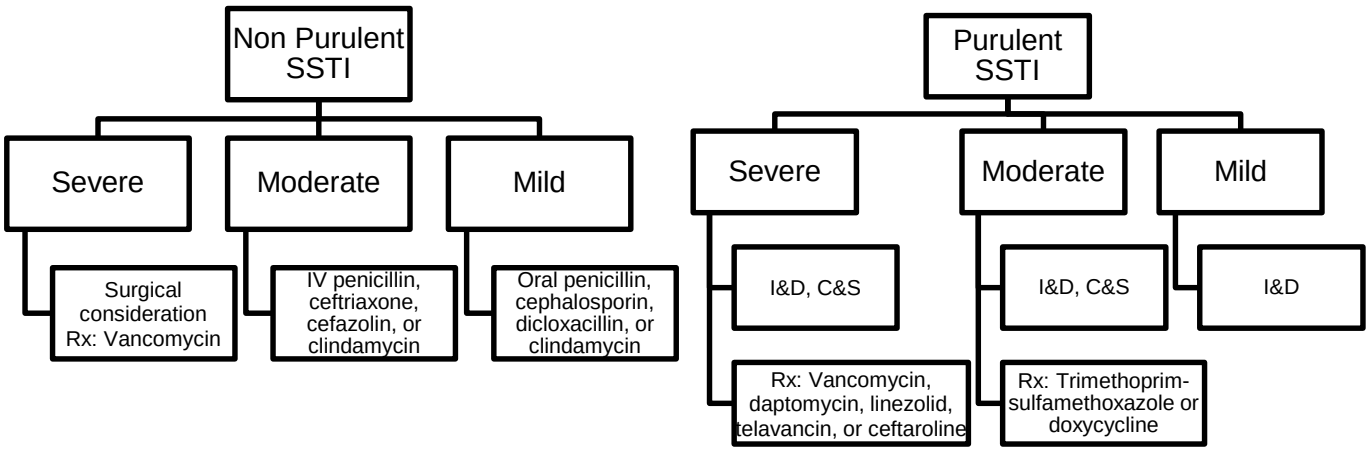
The Infectious Diseases Society of America (IDSA) developed the 'Practice Guidelines for the Management of SSTIs: 2014 Update by the IDSA'.[82] A summary of the management recommendations are shown in Figure 1.4. The IDSA asserts that incision, evacuation of pus and debris, and probing the cavity to break up loculations is the primary treatment of purulent staphylococcal skin abscesses. Patients with abscesses caused by *S. aureus* are frequently cured by incision and drainage (I&D) alone.[83] This contention has been supported by several observational studies that described patients who received inappropriate MRSA antimicrobials or no antimicrobials were cured, nevertheless.[14,50,84,85] In a randomized clinical trial, patients with uncomplicated SSTIs were randomized after I&D to receive either cephalexin or placebo. Despite 88% of isolates being MRSA, cure rates remained high and did not significantly differ between the two groups (84.1% vs. 90.5%).[85]

Although I&D alone may be sufficient for treatment in many SSTI cases, there are likely to be subgroups in which antibiotic therapy is needed. The 2014 IDSA guideline recommends adjunctive antibiotics for purulent SSTIs in patients who have failed I&D plus oral antibiotics, those with systemic signs of infection such as temperature >38°C, tachycardia (heart rate >90 beats per minute), tachypnea (respiratory rate >24 breaths per minute) or abnormal white blood cell count (<12 000 or <400 cells/μL), or patients who are immunocompromised.[82] Furthermore, several studies have shown that appropriate MRSA therapy in

addition to I&D has been associated with higher cure rates.[69,86-91] In a retrospective study of hospitalized patients with CA-MRSA SSTIs, treatment failure occurred less frequently when MRSA-appropriate antibiotics were administered within 48 hours. compared to when MRSA-inappropriate therapy was used (5% vs. 13%; *p*=0.001).[92] A recent study of treatment outcomes of patients with MRSA SSTIs found I&D with adjunctive antibiotics was the only predictor of treatment success. Patients treated with appropriate antibiotics demonstrated significantly higher cure rates compared to those who received inappropriate therapy (91.3% vs. 75.4%; $p<0.05$).[90]

The efficacy of various antibiotics in the treatment of CA-MRSA SSTIs in the clinic setting is not well defined. However, clindamycin and trimethoprim-sulfamethoxazole (TMP-SMX) are recommended due to their relatively low cost and good activity against both CA-MRSA and MSSA strains. A recent randomized clinical trial compared clindamycin and TMP-SMX for the treatment of uncomplicated skin infections and found no significant differences between the two groups with respect to efficacy, and safety outcomes.[84] Further comparative effectiveness studies are required to identify which antibiotic, if any, is optimal in the treatment of staphylococcal skin infections.

**Figure 1.4** Overview of the management of SSTIs[82]




SSTI=skin and soft tissue infection; Rx=antimicrobial therapy; I&D=incision and drainage; C&S=culture and susceptibility testing; “severe”=patients who have failed incision and drainage plus oral antibiotics or those with systemic signs of infection such as temperature >38°C, tachycardia (heart rate >90 beats per minute), tachypnea (respiratory rate >24 breaths per minute) or abnormal white blood cell count (<12 000 or <400 cells/µL), or immunocompromised patients; “moderate”=patients with systemic signs of infection; “mild”=typical cellulitis/erysipelas or purulent SSTI with no systemic signs of infection.

*Antimicrobial Resistance in S. aureus*

Resistance to antibiotics is common among infections caused by *S. aureus.*[2,16,93,94] The most clinically important has been the development of methicillin resistance. Methicillin resistance also infers resistance to most all of the β-lactam class, which are the most effective against *S. aureus*. Methicillin resistance is usually due to the horizontal gene transfer of a mobile genetic element, SCCmec, carrying the *mecA* gene into the *S. aureus* chromosome. The *mecA* gene codes for a penicillin binding protein (PBP2a), which results in decreased affinity to methicillin and all β-lactam antibiotics.[95] As a result, methicillin (and other β-lactam antimicrobials) cannot efficiently bind to the bacterial cell, which results in reduced capacity to inhibit cell-wall synthesis.

Non β-lactam antibiotics are commonly used for the treatment of *S. aureus* in the outpatient setting. Reported susceptibility profiles of USA300 strains are summarized in Table 1.5. USA300 MRSA strains are usually susceptible to trimethoprim-sulfamethoxazole, doxycycline, rifampin, and gentamicin; whereas, the majority is resistant to erythromycin (50-95%).[96] Overall, USA300 isolates are typically susceptible to most antimicrobial classes; however, reports of antimicrobial resistance have been increasingly reported.[16,33,97-99]

**Table 1.5** Antimicrobial susceptibility profiles of CA-MRSA

| Study | Clone | AG | CLIN | ERY | FA | FQ | MUP | RIF | TET | TMP/SMX | VAN |
|---|---|---|---|---|---|---|---|---|---|---|---|
| McDougal et al.[118] | USA300 | 99 | 93 | n/a | n/a | n/a | 97 | n/a | 91 | 99 | n/a |
| Mendes et al.[98] | USA300 | n/a | 93.5 | 1.8 | m/a | 57.4 | n/a | n/a | 91.3 | 99.6 | 100 |
| David MZ et al.[27] | USA400 | 95.8 | 42.5 | 40 | n/a | 93.3 | n/a | 97.5 | n/a | 100 | 100 |
| Huang et al.[42] | USA300 | 100 | 96 | 7 | n/a | 53 | n/a | 100 | 80 | 100 | 100 |
| Luna VA et al.[119] | USA300 | n/a | 96 | 6 | n/a | n/a | n/a | n/a | n/a | n/a | n/a |

CA-MRSA=community-associated methicillin resistant *S. aureus*; AG=aminoglycoside; CLIN=clindamycin; ERY=erythromycin; FA=fusidic acid; FQ=fluoroquinolones; MUP=mupirocin; RIF=rifampin; TET=tetracycline; TMP/SMX=trimethoprim/sulfamethoxazole; VAN=vancomycin

Since 2001, USA300 isolates have become increasingly resistant to fluoroquinolones. In 2006, approximately 54% of the Active Bacterial Core surveillance isolates were resistant to levofloxacin.[100] Previous studies have described numerous fluoroquinolone resistance mechanisms. These include chromosomal mutations in the *gyrA* and *grlA* genes, and the overexpression of a membrane-associated multidrug efflux pump, *norA*.[101]

Resistance mechanisms for most other antimicrobials are often plasmid-mediated and associated with transposons or insertion sequences.[102-104] Among isolates with a susceptibility pattern that is resistant to penicillin, oxacillin, and erythromycin, two plasmids have been described: 1) a small (3.1 kb) cryptic

plasmid, and 2) a mosaic plasmid (27 kb) that harbors several antimicrobial resistance determinants, including erythromycin resistance (*msrA*) and penicillin (*blaZ*).[33,105,106] A plasmid (4.4 kb) with the *tetK* gene has been reported among USA300 strains that are tetracycline resistant but susceptible to doxycycline and minocycline.[33,102,107,108]

Clindamycin resistance among *S. aureus* in the U.S. has been reported to range from 2 to 7%.[77] Resistance to clindamycin can be constitutive or inducible (i.e., inducible macrolide-lincosamide-streptogramin [$MLS_B$] resistance). Due to reports of clindamycin treatment failures, the CDC recommends that isolates that test resistant to erythromycin and susceptible to clindamycin be further tested for inducible clindamycin resistance using the D-test.[16,98,99,109,110] Isolates with clindamycin resistance have been reported to carry a small plasmid (2.6 kb) harboring the *ermC* gene.[33]

With increasing pressure to prevent MRSA infections, there has been an increased use of mupirocin as a decolonization strategy. Prior studies have shown that the widespread use of mupirocin can lead to an increase in mupirocin resistance.[111,112] High-level mupirocin resistance in *S. aureus* is mediated by a plasmid-encoded *mupA* gene. The *mupA* gene is found on conjugative plasmids that carry multiple resistance determinants for other classes of antimicrobial agents. High-level resistance has been associated with decolonization failure, and increased resistance rates have been associated with increased mupirocin use.

Low-level mupirocin resistance is mediated via a mutation in the native *ileS* gene.[111,113,114]

Recent studies have described the increasing incidence of multidrug resistance among community-associated *S. aureus* strains.[16,98] Multidrug resistance was first described in the first complete draft genome of an USA300 CA-MRSA isolate.[102] This strain had accumulated resistance to β-lactams, fluoroquinolones, tetracycline, macrolide, clindamycin, and mupirocin. It was discovered that the resistance determinants for 3 of these agents (macrolide, clindamycin, and mupirocin) were located on a large conjugative plasmid, pUSA03, carrying *ermC* and *mupA*. The pUSA03 plasmid is related to an older plasmid family that contains multiple copies of the insertion element IS*257* which serve as integration sites for mobile elements that can carry various resistance determinants. These determinants include the *aa6'-aph2"*, which confers resistance to aminoglycosides; *dfrA* which confers high-level resistance to trimethoprim; *mupA* which confers high-level mupirocin resistance; and *vanA* which confers vancomycin resistance.[102,104]

Since its discovery, *S. aureus* has acquired resistance to virtually all antibiotics. More recently, reports of vancomycin resistant *S. aureus* and strains resistant to newer antibiotics such as linezolid, daptomycin, and ceftaroline have been reported.[115-117] Multidrug-resistant MRSA strains remain an alarming situation.

*Virulence factors in S. aureus*

*S. aureus* is a major human pathogen responsible for a wide array of infections. Staphylococcal infections can cause a broad range of clinical manifestations and can usually be subdivided into three main groups: superficial lesions (e.g., SSTIs), invasive infections (e.g., pneumonia, endocarditis, and osteomyelitis), and toxinoses (e.g. food poisoning, toxic shock syndrome). The ability of *S. aureus* to cause infections is dependent upon its extensive armamentarium of virulence factors. Host invasion by *S. aureus* and the establishment of infection involve several essential steps: 1) binding to the cell surface, 2) escape from the host immune system, and 3) dissemination and tissue invasion. These virulence factors can be broadly grouped according to three main roles during the infection process: (1) adhesins, which facilitate adherence to host tissues, (2) toxins, which cause tissue damage to the host; and (3) evasins, which interfere with host immune function (Table 1.6).[9,94,120,121]

**Table 1.6** *S. aureus* virulence factors involved with infection

| Type of virulence factor | Factors | Genes |
|---|---|---|
| Adhesins (MSCRAMM) | Clumping factors, fibronectin- finbrinogen-binding proteins, collagen, and bone sialoprotein-binding proteins | *clfA, clfB, fnbA, fnbB, can, sdr, bbp, eap, isdA, emp, ebh* |
| Toxins | Leukocidins (PVL, β toxin, gamma toxin, delta toxin, PSMα), capsular polysaccharides, protein A, phenol-soluble modulins, enterotoxins, toxic shock syndrome toxin-1, exfoliative toxins A and B, α-toxin | *Luk-S-PV, lukF-PV, hlg, cap5 and cap8 gene clusters, spa, chp, eap, psm-α gene cluster* |
| Evasins | Proteases, lipases, nucleases, hyaluronate lyase, phospholipase C, metalloproteases, catalase, superoxide dismutase | *V8, hysA, hla, plc, sepA, dlt, mprF* |

MSCRAMM=microbial, surface components recognizing adhesive matrix molecules; PVL=panton valentine leukocidin; PSM=phenol soluble modulin

Staphylococcal virulence molecules include those that inhibit neutrophil recruitment, promote direct lysis of neutrophils, and those that are involved in the formation of the fibrin capsule surrounding the abscess.

*S. aureus* demonstrates an abundance of mechanisms to evade killing by phagocytes.[122] *S. aureus* may hide from recognition by producing protective coats, such as capsular polysaccharide or biofilm produced by polysaccharide intercellular adhesion (PIA).[122-125] It can also secrete specific molecules to block phagocyte receptor function. These bacteria have also been shown to use mechanisms to decrease the efficiency of antimicrobial activity within the phagosome, that can account for postphagocytic survival. *S. aureus* has the capacity to produce a molecule called CHIPS (chemotaxis inhibitory protein of *S. aureus*) that blocks receptor-mediated recognition of formylated peptides.[122,126]

Furthermore, other molecules secreted by *S. aureus* can block the host complement system, which results in reduced phagotysois after opsonization.

After phagocytosis, *S. aureus* produces a diverse array of immune evasion molecules. These include catalase and superoxide dismutase to eliminate reactive oxygen species. Non-specific proteases are secreted to digest any protein-based antimicrobial effector. In addition, *S. aureus* can react in the presence of antimicrobial peptides (AMPs) by an up-regulation mechanism to interfere with the cationic activity of AMP.[122,127]

*S. aureus* produces several virulence factors that contribute to the formation of abscesses and host immune evasion. The roles of each of these virulence factors are discussed.

Panton valentine leukocidin (PVL) is an exotoxin that is comprised of two subunits, LukS-PV and LukF-PV, encoded by ΦSa2.[128] These subunits bind to specific membrane receptors to form pores in the membrane of host leukocytes, and at high concentrations, causes lysis of white blood cells. However, studies have yielded conflicting results of its role in disease severity. In the early 1990's, PVL was epidemiologically linked with *S. aureus* skin infections and with the enhanced virulence of CA-MRSA infections.[129-132] In a meta-analysis, the presence of PVL was associated with abscesses and furuncles (odds ratio 10.5; 95% CI 7.4-14.9).[133] However, the extent of PVL production was not associated with more severe disease. Evidence from animal models does not conclusively link PVL and the pathogenesis of *S. aureus* skin infections. In mice, PVL-negative

USA300 *S. aureus* strains demonstrated no difference to PVL-positive strains in a skin infection model.[134,135] It was later discovered that mouse neutrophils are resistant to the toxic effects of PVL compared to humans, thus does not serve as an optimal model.[136] Comparatively, rabbit models demonstrated the potential toxic effects of PVL. A recent study compared isogenic PVL-knockout mutants, and found that PVL-positive MRSA strains exert toxic effects on keritonocytes that facilitate local spread and inflammation.[129] However, in a another rabbit model study, PVL-positive and PVL-negative strains produced comparable severity in disease.[137] Currently, the contribution of PVL to *S. aureus* pathogenesis remains inconclusive.

Phenol soluble modulin-like peptides (PSMs) are a group of small proteins in *S. aureus* that has the capacity to lyse host cells including neutrophils and erythrocytes.[9,10,120,138] High expression of PSMs has been associated with enhanced virulence of MRSA. In a mouse abscess model, PSM deletion resulted in significantly less severe skin lesions. The level of PSM production was found to be significantly higher in USA300 MRSA clones than other MRSA clones. In a rabbit skin infection model, α-hemolysin, PSM, and *agr*, were associated as main contributors to the pathogenicity of MRSA isolates.[139]

Another toxin, α-toxin (α-hemolysin, Hla) and its role in *S. aureus* virulence has been well studied. α-hemolysin has the capacity to form pores in various human cells, including red blood cells, epithelial cells, fibroblasts, and monocytes.[138,140,141] In a rabbit skin infection model, the expression level of α-

hemolysin was correlated with virulence. High levels of α-hemolysin have also been associated with severe skin infections due to an ST93 MRSA clone.[142] Early vaccination studies demonstrated that mice that were vaccinated against α-hemolysin were associated with less severe skin disease.[143]

More recently, the pathogencity of USA300 MRSA strains has been associated with the arginine catabolic mobile element (ACME).[144] ACME is adjacent to the SCCmecIV and contains two potential virulence factors including a cluster of *arc* genes that encode an arginine deiminase pathway and Opp-3, which encodes an oligopeptide permease operon.[102,145,146] Specifically, the *speG* gene within the ACME locus was found to be associated with increased resistance to polyamines that are produced on the skin, which are toxic to other *S. aureus* strains. In addition, a rabbit bacteremia model showed that the deletion of ACME (but not SCCmec) was shown to decrease fitness of USA300 MRSA strains.[144] This supported a key advantage for ACME containing strains for skin colonization and infection.

*Pathogenesis of S. aureus SSTIs*

*S. aureus* is responsible for 85 to 95% of all skin infections. The propensity for *S. aureus* to infect the skin involves a balance between the host's cutaneous immune defense mechanisms and virulence factors of the pathogen.

The skin is the first line of defense against invading pathogens. The skin provides a physical barrier to prevent bacteria from entering into deeper layers of tissues and/or invasion to other internal organ systems. Therefore, breach of the skin allows entry of pathogenic organisms into the tissue and initiates an immune response to the site of infection.

Approximately 65% of *S. aureus* SSTIs present as pyogenic abscesses that form in the dermis, epidermis, or subcutaneous tissue and are frequently present with surrounding cellulitis.[83] The overall structure of *S. aureus* abscesses begins as an acute localized host inflammatory response.[147] The center of the abscess comprises of an exudate composed of viable and necrotic polymorphonucleocytes (PMNs), tissue debris, fibrin, and bacteria. The abscess maturation is then accompanied by the formation of a fibrous capsule at the periphery due to fibroblastic proliferation and tissue repair. Keratinocytes on the skin possess receptors that detect patterns in invading microbes which signal the pro-inflammatory response.[148,149]

Neutrophil recruitment is arguably the most important cellular defense against *S. aureus.* Neutrophils play an important role in the formation and resolution of *S. aureus* abscesses. Findings in mice and humans have suggested important roles for IL-1 and IL-17 in host defense against cutaneous *S. aureus* infections.[151-153] Neutrophils are rapidly recruited to the infection site to initiate the phagocytosis process to remove invading organisms. Receptors on the surface of host neutrophils and other phagocytes play an essential role in recognizing bacteria by promoting chemotaxis and activation. Bacteria have a multitude of surface molecules that interact with receptors on the surface of neutrophils. These pattern recognition receptors (e.g. Toll-like receptors and CD14) activate signal transduction pathways that lead to bactericidal activity.[148,154-156] This occurs primarily by opsonization of bacteria with antibody and complement. Antibodies bind to the epitopes on the surface of bacteria. These antibodies that are bound to the bacterial surface are then recognized by neutrophil receptors specific for the Fc region. The ingested bacteria are engulfed within membrane-bound vacuoles (phagosomes). Following polymorphonucleocytes (PMN) phagocytosis, signal transduction of various bactericidal mechanisms is initiated, including the production of superoxide radicals and other reactive oxygen species (ROS), production of antimicrobial peptides (AMP) that have activity against *S. aureus*, proteins, and degradative enzymes.[122,127,150,157,158]

*S. aureus colonization*

*S. aureus* is normally a commensal and colonizer of the skin and mucosa of humans and several animal species.[94,159,160]. The primary carriage site for *S. aureus* in humans is the anterior nares (foremost parts of the nose, covered by fully keratinized squamous epithelium containing hair follicles).[161,162] But, the skin, throat, gastrointestinal tract, groin, anus, and the urogenital tract in women have all been identified as niches. Longitudinal studies have shown that 12-30% of individuals are persistent *S. aureus* carriers and approximately 30% are intermittent carriers of *S. aureus.* Overall MRSA carriage rates in the community setting are still low (~1.5%), but seem to be rising rapidly in certain regions.[163,164]

The mechanisms leading to *S. aureus* nasal carriage are multi-factorial. Bacterial factors (e.g. staphylococcal toxins and adhesion molecules), environmental factors (e.g. hospitalization and prisons), as well as host susceptibility factors (e.g. immune suppression, loss of normal skin barrier, malignancies) all play a role in successful *S. aureus* coloniziation.[60,165,166] Nouwen et al. conducted a study where subjects (non-carriers and persistent carriers) were inoculated with a mixture of *S. aureus* strains. This study demonstrated that non-carriers quickly eliminated the inoculated *S. aureus* strains, whereas most persistent carriers selected their original resident *S. aureus* strain from the inoculation mixture.[160] *S. aureus* carriage rates have been reported to vary depending on age, race, ethnicity, geographic location, and body niche. Carriage has shown to be higher among patients with diabetes mellitus, end-stage renal

disease undergoing hemodialysis, end stage liver disease, HIV, skin disease, obesity, and history of a cerebrovascular accident. Environmental factors such as hospitalization are important risk factors for nasal carriage state. In addition, community households have been determined to be a critical reservoir for *S. aureus*.[74,75] Peacock et al. found concordant carrier states between mothers and their children. Bogaert et al. also found that most mothers carry the same strain as their children, further indicating that carriage strains are transmitted to close contacts.[165]

Nasal colonization of *S. aureus* has been described to occur in several steps. First, the nose has to come in contact with *S. aureus*. Second, *S. aureus* needs to adhere to certain receptors in the nasal niche. Third, *S. aureus* needs to overcome the host defenses. Finally, *S. aureus* must be able to propagate in the nose.

Colonization is an important risk factor for invasive disease and source of spread of *S. aureus* among individuals.[163,167] Several older studies have investigated the relationship between *S. aureus* nasal carriage and skin infections, including furunculosis, impetigo, sycosis barbae, stye, osteomyelitis, infective endocarditis, and respiratory tract infections (cystic fibrosis).[168-171] On average, 80% (range 42–100%) of those with skin lesions were *S. aureus* nasal carriers, and 65% (range 29–88%) had the same phage type in the nose and lesion. In a prospective study, Ellis et al. found a higher risk (relative risk 3.1, 95% CI 1.5-6.5)

for nasal carriers of MRSA to acquire a community-associated MRSA infection (i.e., cellulitis, abscess).[172]

There is controversy as to whether all *S. aureus* strains have equal disease invoking potential or whether invasive disease is associated with particularly virulent genotypes. While it has been well established that nasal colonization is a predisposing factor for noscomial infections, the potential role for opportunistic infections in other sites and syndromes are unknown. Few studies have investigated the potential of commensal *S. aureus* strains and their capacity to cause invasive disease.[173-176] In a study of 219 bacteremic patients, *S. aureus* isolated from blood could not be distinguished by PFGE from concomitantly carried nasal bacteria in the majority of cases.[177] Conversely, in a major study, Feil et al. found no significant differences in the distribution of genotypes based on MLST between strains isolated from carriers and those from patients with invasive disease.[39] This study suggested that there was no evidence for the existence of hyper-virulent *S. aureus* clones. A subsequent microarray analysis comparing community associated invasive isolates and 100 nasal carriage isolates did not reveal differences in lineage or gene content associated with pathogenic potential.[178] Sabat et al. found that there was also no significant difference in the prevalence of genes encoding for adhesive proteins (*SdrC*, *SdrD*, and *SdrE*) between commensal (n=259) and invasive isolates (n=141) from different countries.[179] By contrast, Melles et al. reported that subclusters of strains have different proportions of those that were isolated from invasive infections. This study

also revealed that all major clusters contained both invasive and non-invsaive isolates. The investigators concluded that given the right clinical condition, essentially any *S. aureus* genotype carried by humans can transform into an invasive pathogen, although certain clones may be more virulent than others.[180] Contemporary studies to better understand the ecology, pathogenesis, and epidemiology of *S. aureus* nasal carriage and its relation to skin infections are needed to develop and target preventive measures.

# CHAPTER TWO

## Translational science of bacterial whole genome sequencing

*Application of bacterial whole genome sequencing (WGS)*

WGS is a process that yields all of the available DNA information from a genome. Over the last decade, significant advances have been made with next generation sequencing technology (NGS). NGS systems are powerful platforms that allow for massively parallel sequencing reactions and are capable of analyzing billions of sequencing reactions simultaneously. The first draft of the human genome sequence took ~13 years and over $3 billion.[25,26] With the advent of NGS, the complete human genome sequence can now be assembled in less than 5 days at a cost of $2,000. NGS has the potential to revolutionize fields such as personalized medicine, genetic diseases, and clinical diagnostics.

WGS of bacteria is poised to transform the practice of clinical microbiology and management of infectious diseases. The rapidly falling cost and turnaround time indicate that microbial WGS will become a viable technology in diagnostic and reference laboratories in the future. WGS has started to transform our understanding of the evolution of pathogens, identify the global spread of antimicrobial resistance, and represents the most significant advancement in diagnostic microbiology and surveillance since *in vitro* culture. In principle, WGS data contains all the information a clinician would need to direct treatment and provide public health surveillance in a single step. Over the last several years,

many studies have demonstrated the value of WGS for species identification, antimicrobial susceptibility and virulence prediction, and outbreak detection.

*WGS for controlling antimicrobial resistance*

WGS has been found to have numerous applications in controlling antimicrobial resistance ranging from the development of novel antibiotics, antimicrobial resistance surveillance, and antimicrobial susceptibility testing in the practice setting.[181,182]

WGS has been instrumental in reconstructing the evolution of antimicrobial resistance and elucidating novel mechanisms of resistance. In a recent study, Long et al. used WGS to identify two novel amino acid altering mutations within the ceftaroline-binding pocket of PBP2a that were associated with ceftaroline resistance.[117] Peleg et al. characterized the genetic evolution of daptomycin resistance in *S. aureus.* The investigators identified novel point mutations in genes coding for membrane phospholipids (*cls2* and *pgsA*) that were associated with the development of daptomycin resistance.[183] Furthermore, Alam et al. used WGS to identify genetic variants associated with vancomycin-intermediate *S. aureus* (VISA) strains. The study also developed a model to predict VISA based on the presence of these mutations in a set of candidate genes.[184]

WGS has also been pivotal in validating and improving accuracy of current diagnostic assays by identifying novel resistance mechanisms. For instance, WGS led to the discovery of *mecC* (a homologue to *mecA,* a methicillin-resistance gene in *S. aureus*). This led to the redesign of clinical PCR (polymerase chain reaction) arrays to avoid false-negatives of MRSA detection.[185]

*S. aureus* infections are associated with significant morbidity, mortality, and health care costs. Time to appropriate diagnoses and treatment is a vital component of health outcomes. Prior studies have shown that every hour of inappropriate antibiotics substantially increases mortality among septic patients.[186] However, current culture and sensitivity practices take up to five days for results. The use of rapid susceptibility testing to guide appropriate treatment of *S. aureus* infections is sorely needed. Because WGS extracts the sum of all the bacterial genetic information, it can potentially determine a plethora of phenotypic characteristics, including antimicrobial resistance determinants. WGS can potentially identify the presence and absence of antimicrobial resistance genes and chromosomal mutations associated with reduced antimicrobial susceptibility.[181,187]

Several studies have found concordance between the genotype derived by WGS and a phenotypically derived antibiogram (Table 2.1).[188-192] Koser et al. compared antimicrobial susceptibilities of 13 antimicrobials in 14 clinical strains. The study found complete concordance between genotype-phenotype profiles for antimicrobial resistance.[191] Eyre et al. evaluated isolates from a hospital outbreak affecting eight patients. All outbreak cases were *mecA* positive and harbored two plasmids that contained the *blaZ* and *tetK* genes, encoding for penicillin and tetracycline resistance, that aligned with the phenotype.[189] Similarly, in a pilot study among 13 CA-MRSA isolates, Lee et al. found complete concordance of genotypic characteristics and its respective antimicrobial susceptibility phenotype.[192] In the

largest study to date, Gordon et al. sought to develop a method for genotypic prediction of antimicrobial susceptibilities from whole genome sequences of 501 *S. aureus* strains collected from two hospitals in England. This study compared the routine antimicrobial susceptibility testing results of 12 common antibiotics to WGS data to develop the panel of antimicrobial resistance determinants. This panel was subsequently used on a "validation set" of 491 unrelated *S. aureus* strains, which yielded an overall sensitivity and specificity of 0.97 (95% CI, 0.95-0.98) and 0.99 (95% CI, 0.99-1), respectively. The very major discrepancy rate was 1.2% and a major discrepancy rate was 0.3%.[190] These error rates were comparable with error rates of current phenotypic tests, and within acceptable limits set by the Food and Drug Administration (FDA) for new susceptibility testing. Finally, Bradley et al. implemented a rapid antibiotic resistance detection tool ("Mykrobe predictor") on an independent set of 470 *S. aureus* strains. This method had comparable error rates when compared to standard antibiotic susceptibility methods, with a sensitivity and specificity of 99.1% and 99.6%, respectively.[193]

**Table 2.1** Summary of studies evaluating WGS to detect antimicrobial resistance

| Study | Genotype-Phenotype Concordance |
|---|---|
| Koser et al. (n=14) | 100% concordance |
| Eyre et al. (n=8) | 100% concordance |
| Lee et al. (n=13) | 100% concordance |
| Gordon et al. (n=501 derivation; n=491 validation) | Sensitivity=97%; specificity=99%<br>VME=1.2%; ME=0.3% |
| Bradley et al. (n=470) | Sensitivity=99.1%; specificity=99.6% |

WGS=whole genome sequencing; VME=very major error; ME=major error

Overall, several studies have documented the potential value of antimicrobial resistance profile predictions of *S. aureus* based on WGS data. However, the distribution and detection of antimicrobial resistance determinants among *S. aureus* in the outpatient setting is unknown. Because the majority of CA-MRSA cases occur in the outpatient setting, studies evaluating the feasibility of using WGS data from community-associated *S. aureus* isolated from outpatient profiles are needed. Before WGS data can be used in the clinical setting for antimicrobial resistance prediction, multiple larger studies using diverse sets of unrelated *S. aureus* isolates are needed to validate the concordance of genotypic profiles relative to the phenotypically derived antibiogram. Furthermore, it is unclear whether current breakpoints (determined by *in vitro* MICs) or the presence of specific genetic mechanisms is more predictive of antimicrobial activity.

*WGS to predict pathogenicity and virulence*

There is a growing body of evidence that suggests that the pathogenicity and virulence of bacteria can be predicted by microbial genotyping.[105,156,175,178,194] Studies evaluating pathogens such as *Pseudomonas aeruginosa, Salmonella enterica,* and *S. aureus* have identified important virulence genes by comparing serotypes of either less virulent or avirulent strains.[176,195,196] Fowler et al. demonstrated significant association between specific clonal complexes (CC) and severity of infection in *S. aureus.* Among 371 *S. aureus* strains from diverse clinical settings, CC5 and CC30 were significantly associated with hematogenous complications such as endocarditis, septic arthritis, and vertebral osteomyelitis.[175] Gill et al. evaluated the association between the presence of specific genes among 230 *S. aureus* clinical isolates and clinical outcome using array comparative genomic hybridization. Clinical outcomes were classified as “uncomplicated” (e.g., uncomplicated bacteremia or SSTI) and “complicated” (e.g., bacteremia with hematogenous complications). The investigators identified a total of 226 genes that were associated with severity of infection; 51 were more frequent and 175 less frequent in the complicated infection group.[194]

While the above studies demonstrated the association between CC or presence and absence of genes with *S. aureus* clinical manifestations, these methods might have limited discriminatory power to detect higher resolution genetic changes. Thereby undetected and heterogenous populations may be broadly considered clonal. Recent advances in WGS technologies have offered

new opportunities to study infectious organisms at the single nucleotide level. However, identifying the genetic variants that explain differences in disease remains challenging.

Melles et al. performed a high-throughput analysis called amplification fragment length polymorphism (AFLP) to analyze a collection of 900 *S. aureus* isolates from healthy nasal carriers and from invasive syndromes, including isolates from SSTIs and bacteremia. AFLP is a DNA technology that generates isolate specific DNA fingerprints by amplifying distinct subsets of genomic fragments by PCRs. Cluster analysis revealed sub-clusters of strains with differential degrees of pathogenicity. Subpopulations harboring specific toxin patterns were found in *S. aureus* strains associated with SSTIs. In contrast, carriage strains were found in distinct homogenous clusters, demonstrating that some clones are more virulent than others.[180]

Although disease may follow entry of commensal *S. aureus* into the infection site through compromised epithelia, another important possibility is that subtle changes in the pathogen through host-pathogen interactions can precipitate the onset of disease. Young et al. used high-throughput genome sequencing to longitudinally investigate the genetic changes that occur during the transition from nasal carriage to a fatal blood stream infection.[197] Of the recruited 360 *S. aureus* carriers, one patient developed a blood stream infection 15 months after study initiation. The investigators sequenced multiple colonies isolated from nasal swabs at different time points (early and late), and from a blood culture. All isolates were

MSSA, (MLST) ST-15. To compare the evolutionary changes, the investigators contrasted the genetic changes with two asymptomatic *S. aureus* carriers. The study found that the bloodstream *S. aureus* differed by only 8 SNPs from the original nasal carried bacteria. Half of these mutations are associated with truncation of proteins, including a premature stop codon in an *AraC* family transcriptional regulator. When compared with the two asymptomatic carriers, the analysis supported the notion that a significant excess of protein truncating mutations occurred on two branches separating genomes sampled from nasal carriage from those sampled from the invasive bloodstream infection ($p$=0.001). This study demonstrated that bacterial diversity is limited yet detected by WGS approaches. In addition, loss-of-function point mutations that truncate the amino acid sequences may play an important role in *S. aureus* pathogenesis.[197,198]

To further the notion that minimal mutations affect *S. aureus* virulence, Kennedy et al. evaluated 10 CA-MRSA USA300 clones using comparative WGS. The investigators identified two MRSA clones containing 21 and 40 SNPs in the core genome that had significantly higher survival rates in a mouse sepsis model, compared with FPR3757.[199] This study suggested that the difference in virulence or pathogenicity were due to subtle changes (SNPs) within key virulence factor genes and not necessarily the large scale gain or loss of entire genes.

Recently, Laabei et al. performed the first study to predict MRSA toxicity from WGS.[200] A genome wide association study (GWAS) was conducted to identify specific loci and putative networks associated with toxicity of 90 MRSA isolates.

The phenotypic traits were evaluated using fibronectin and fibrinogen binding assays to assess the ability for strains to bind human tissues, and toxicity assays to evaluate the gross lytic activity. Strains were classified as low, medium, or high toxicity. Overall, a total of 122 SNPs and indels were identified to be associated with toxicity. These variants were distributed across the genome among MGE, genes involved in metabolism, intergenic regions, and hypothetical genes. The identified genetic signatures (SNPs and indels) associated with toxicity was used to build a class-prediction model that demonstrated a moderate degree of accuracy in predicting high and low toxicity strains.

High-throughput WGS allows a more comprehensive study of the combination of genetic signatures, regulatory networks, and virulence factors that affect disease outcomes. While significant progress has been made, no studies, to date, have used a WGS approach in evaluating genetic signatures associated with clinical manifestations of staphylococcal infections.

# CHAPTER THREE

## Specific aims

### Specific Aim 1

To describe the clinical epidemiology, antimicrobial resistance determinants, and genomes of *S. aureus* strains in the South Texas primary care setting

*Sub-aim 1a*: Describe the population structure of community-associated *S. aureus* isolates

*Sub-aim 1b*: Describe the epidemiologic factors associated with multidrug-resistant community-associated *S. aureus* isolates

*Sub-aim 1c*: Apply WGS to describe the diversity and distribution of resistance mechanisms among community-associated *S. aureus* isolates

*Sub-aim 1d*: Determine the extent to which the genotype is predictive of antimicrobial resistance

### Specific Aim 2

To describe the genomic heterogeneity of *S. aureus* associated with colonization and SSTIs in the South Texas primary care setting

*Sub-aim 2a*: Describe the epidemiologic factors associated with *S. aureus* SSTIs and nasal colonization

*Sub-aim 2b*: Apply WGS to describe the diversity and distribution of virulence mechanisms among community-associated *S. aureus* isolates

*Sub-aim 2c*: Identify *S. aureus* genetic signatures associated with SSTI isolates compared to colonization isolates

*Sub-aim 2d*: Derive a predictive model to identify *S. aureus* isolates associated with SSTI

# CHAPTER FOUR

# Materials and methods

*Materials and methods*

Materials and methods described in this chapter are commonly used in the subsequent study results chapters. Methods specific to particular parts of the analyses are described in more detail in the relevant chapters.

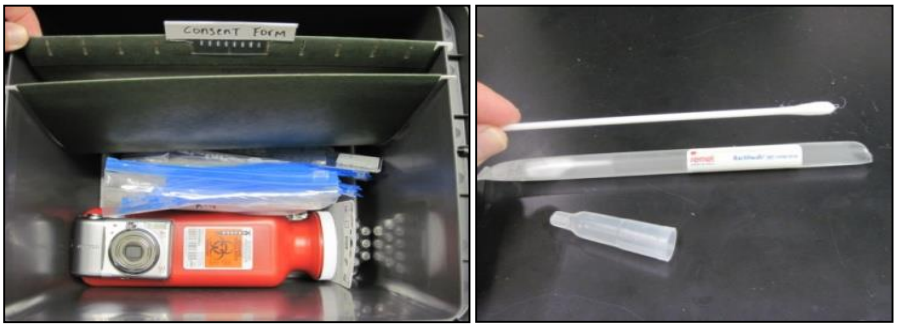
*S. aureus SSTI study population*

Bacterial samples and patient information used in the investigations were from a multisite, prospective, community-based, observational cohort study led by Dr. Christopher Frei in collaboration with the South Texas Ambulatory Research Network (STARNet), a practice based research network (PBRN) composed of 108 urban, suburban, and rural primary care clinics distributed throughout the South Texas region.[49,50] Health care providers at 17 STARNet clinics prospectively enrolled patients for this study from 2007 to 2013. Patients were eligible for study enrollment if they provided informed consent, were 18 years of age or older, presented to one of the participating clinics with a SSTI, and if their managing clinician suspected *S. aureus.* Patients were excluded if they were pregnant, incarcerated, or had impaired decision-making capacity. The subsequent analyses conducted with this study were limited to patients and samples confirmed to be *S. aureus* via microbiological testing described below. A total of 112 from a total of

175 *S. aureus* SSTI cases were selected for the analyses conducted in this dissertation. The University of Texas Health Science Center Institutional Review Board granted approval for this study.

Participating clinics were provided with English and Spanish informed consent forms, a digital camera, a portable bio-hazard container, and individually study kits containing a 'clinical information card', 15cm plastic ruler (Accu Ruler®, Macon, GA), and a rayon tipped wound swab (Bactiswab®, Remel, Lenexa, KS). Modified Stuart's Transport Media was enclosed in a crushable ampule located at the base of each swab casing, near the swab collecting tip.

**Figure 4.1** Study Kits




Clinicians were instructed to obtain informed consent from patients, record demographic and clinical information on the card, obtain a wound culture, crush the ampule to release the media onto the swab tip for preservation during transport, and capture digital pictures of the infection site. The patient data card included fields for the following information: gender, race, ethnicity, diabetes

history, health care related work history, skin infection history, height, weight, health insurance category, infection diameter, incision and drainage procedures received, antibiotics prescribed, and plans for follow up. Specimens were collected and stored in CryoBeads at -80°C for further testing. Additional follow-up information was requested to be collected via medical chart review after the initial clinic visit. This information included patient age at time of visit, past medical history (e.g., peripheral vascular disease, chronic non-infectious skin disorder, HIV/AIDS, cancer, actively receiving chemotherapy, immunosuppressed at time of visit), type of infection (e.g., furuncle, abscess, cellulitis, other), and events occurring within 90 days of initial clinic visit.

*S. aureus nasal colonization study population*

Bacterial samples and patient information were collected from a subsequent multisite, prospective, community-based, observational cohort study within select participating STARNet clinics. Health care providers prospectively enrolled patients for this study from March 2015 to May 2015. Patients were eligible for study enrollment if they provided informed consent, were 18 years of age or older, and if they presented to one of the participating clinics without an active infection (e.g., SSTI). Patients were excluded if they were pregnant, incarcerated, or had impaired decision-making capacity. A total of 32 colonization cases were selected from the 150 total screened for the analyses conducted in this dissertation. The

University of Texas Health Science Center Institutional Review Board granted approval for this study.

Participating clinics were provided with English and Spanish informed consent forms and individual study kits containing a 'clinical information card' (Appendix A), procedure guide, and a rayon tipped wound swab (BactiSwab®, Remel, Lenexa, KS). Modified Stuart's Transport Media was enclosed in a crushable ampule located at the base of each swab casing, near the swab collecting tip. If the patient consented to participate in the study, the clinic staff were instructed to obtain a nasal swab and collect information as detailed above utilizing the 'clinical information card'. To collect the nasal specimen, the cotton swab was inserted into the patient's nostril (swab tip to be inserted up to 2.5cm from the edge of the nares) and rolled 5 times in the nares. The same swab was inserted into the second nostril and sampling was repeated. The swab was returned to its container and the ampule to release the media was crushed. These swabs were then collected within 24 hours for subsequent microbiological analyses.

*Bacterial identification and antimicrobial susceptibility testing*

Microbiological testing was conducted in Dr. Dallas's Lab (UT Health Science Center at San Antonio), including automated antimicrobial susceptibility testing. Samples were plated onto pre-filled blood agar plates and incubated at 35°C to 37°C for 24 hours, then sub-cultured to MRSA selective agar (MRSA*Selec*t

chromogenic agar plates; Bio-Rad Laboratories, Hercules, CA). Latex agglutination tests (StaphAurex®; Thermo Fisher Scientific, Lenexa, KS), and phenotypic screening tests (cefoxotin) were used for the identification and isolation of MRSA. Vitek2 AST-GP75 cards (bioMerieux, Durham, NC) were used to determine the susceptibility of *S. aureus* study isolates against ciprofloxacin, clindamycin, daptomycin, doxycycline, erythromycin, gentamicin, levofloxacin, linezolid, moxifloxacin, nitrofurantoin, rifampin, tetracycline, trimethoprim-sulfamethoxazole, and vancomycin. Double-disk diffusion tests were performed to identify inducible clindamycin resistance. Mupirocin susceptibility testing was conducted using gradient diffusion testing (Etest, bioMerieux, Durham, NC). Antimicrobial minimum inhibitory concentrations (MICs) were interpreted according to the Clinical and Laboratory Standards Institute documents M100-S12 (2012) and M100-S14 (2014).

*Sequenced isolates*

A collection of 144 single-colony isolates were selected recovered from 144 unique patients. These were selected based on isolates with complete phenotypic data and patient profiles with complete demographic and epidemiologic data.

*DNA extraction*

For DNA extraction, *S*. *aureus* isolates were grown in 3mL tryptic soy broth (TSB) shaking at 37$^{o}$C for 24 hours. Overnight cultures (1.5mL) were harvested by

centrifugation and re-suspended with 200ul of 10 mM Tris-HCl (pH 8) and 2.5 mg/ml of lysozyme solution (Life Technologies, Grand Island, NY). Samples were lysed with 200uL of MagnaPure Bacterial Lysis Buffer (Roche, Indianapolis, IN) and 1 mg Proteinase K (Roche, Indianapolis, IN), then incubated at 37$^{o}$C for 1 hour then 55$^{o}$C for 1 hour. Aliquots of 200uL were transferred to a MagNA Pure 96 Instrument for automated DNA extraction using the Pathogen Universal 200 protocol (Roche Life Science). The quality of the extracted DNA was assessed with the Epoch Microplate Spectrophotometer (Biotek, Winooski, VT) and ran on an automated capillary electrophoresis system (Qiaxcel Advanced System; Qiagen, Valencia, CA). Genomic DNA was quantified using the high-sensitivity double stranded DNA assay on the Qubit 2.0 fluorometer (Invitrogen, Life Technologies, Grand Island, NY). Bacterial DNA extraction was conducted at the Center for Advancing Translational Technologies (South Texas Research Facility, UT Health Science Center at San Antonio).

*WGS*

Sequencing libraries were prepared using the NexteraXT DNA sample preparation kit (Illumina Inc., San Diego, CA) for 250bp paired end reads, following manufacturer's instructions. DNA libraries were sequenced on a MiSeq sequencing instrument (Illumina Inc., San Diego, CA) following manufacturer's instructions. Bacterial sequencing was conducted at the Genome Sequencing

Facility (Greehey Children's Cancer Research Institute, UT Health Science Center at San Antonio).

*Mapping and assembly*

Sequencing data were imported and analyzed using CLC Genomics Workbench 8.1 (Qiagen, Redwood City, CA). Paired-end reads were mapped to a reference strain FPR3757 (accession NC_007793). FPR3757 (length=2,872,769 bp) was the first USA300, ST-8 strain to be completely sequenced; this strain was recovered from a skin abscess, and serves as a good reference strain for our comparative genome analysis. Mapping was conducted using CLC read mapper. The parameters used included the following: mismatch cost=2; linear gap cost; length fraction=0.8; similarity fraction=0.8).

*Visualization of phylogenetic trees*

Display and manipulation of phylogenetic trees was performed using CLC Genomics Workbench version 8.1, Molecular Evolutionary Genetic Analysis (MEGA) version 7, and CSIPhylogeny v1.0a (Center for Genomic Epidemiology).

*Statistical analyses*

All statistical analyses were conducted on SPSS 23.0® (IBM Corp, Armonk, NY, USA). Statistical analyses are discussed in relevant sections in the text.

# CHAPTER FIVE

# Specific aim 1

## Comparative Genomics and Antimicrobial Resistant Determinants of Community-Associated *Staphylococcus aureus* in South Texas

### *Introduction*

Since the discovery of *Staphylococcus aureus,* a major challenge has been its remarkable ability to acquire resistance to antibiotics. The emergence of community-associated methicillin-resistant *S. aureus* (CA-MRSA) has resulted in an epidemic of skin and soft tissue infections (SSTI) in the U.S.[2,37] Whereas multidrug resistance has been well described among traditional hospital-associated methicillin resistant *S. aureus* (MRSA) clones, community-associated *S. aureus* clones have retained susceptibility to non β-lactam antimicrobials including macrolides, tetracyclines, fluoroquinolones, lincosamides, and trimethoprim-sulfamethoxazole. However, the increased use of these antimicrobials could drive the emergence of new multidrug-resistant sub clones of community-associated *S. aureus*, complicating disease management and leading to the development of persistent or recurrent infections. Recent reports of the emergence of multidrug-resistant USA300 strains have occurred in San Francisco, Boston, and New York City.[16,74,96] The main treatment options for MRSA SSTIs include trimethoprim-sulfamethoxazole, clindamycin, and tetracyclines.[82] The

emergence of resistance to these agents among USA300 strains will pose a challenge for treating both community- and health care-associated *S. aureus* infections.

With rapidly advancing technology, microbial whole genome sequencing (WGS) is positioned to soon become a viable option in the routine clinical laboratory.[181,182,201] WGS provides valuable information in reconstructing the evolution of antimicrobial resistance and has the potential to substantially increase the speed of antimicrobial resistance detection in the practice setting.[103,184,202,203] With the ever increasing rise of bacterial drug resistance, the need for rapid and reliable methods to detect its emergence and predict antimicrobial susceptibility has never been more imperative.

This chapter aimed to 1) describe the population structure of community-associated *S. aureus* in South Texas, 2) describe the incidence and epidemiologic factors associated with multidrug-resistant community-associated *S. aureus* isolates in the South Texas primary care setting, 3) use whole genome sequences to describe the diversity and distribution of resistance mechanisms among community-associated *S. aureus* isolates, and 4) determine the extent to which the genotype is predictive of resistance.

## *Methods*

*Study setting and population:*

We performed this investigation using clinical and isolate information from a well-described cohort of patients with *S. aureus* SSTI or nasal colonization in the South Texas primary care setting. Details of this cohort have been described previously.[49] Briefly, this study was conducted in collaboration with ten clinics within the South Texas Ambulatory Research Network (STARNet), a practice-based research network (PBRN) composed of 108 urban, suburban, and rural primary care clinics distributed throughout the South Texas region, from 2007 to 2015.

*Microbiological analysis:*

Samples were plated onto pre-filled blood agar plates and incubated at 35°C to 37°C for 24 hours, then sub-cultured to MRSA selective agar (MRSA*Selec*t chromogenic agar plates; Bio-Rad Laboratories, Hercules, CA). Latex agglutination tests (StaphAurex®; Thermo Fisher Scientific, Lenexa, KS), and phenotypic screening tests (cefoxotin) were used for the identification and isolation of MRSA. Vitek2 AST-GP75 cards (bioMerieux, Durham, NC) were used to determine the susceptibility of *S. aureus* study isolates against ciprofloxacin, clindamycin, daptomycin, doxycycline, erythromycin, gentamicin, levofloxacin, linezolid, moxifloxacin, nitrofurantoin, rifampin, tetracycline, trimethoprim-

sulfamethoxazole, and vancomycin. Double-disk diffusion tests were performed to identify inducible clindamycin resistance. Mupirocin susceptibility testing was conducted using gradient diffusion testing (Etest, bioMerieux, Durham, NC). Antimicrobial minimum inhibitory concentrations (MICs) were interpreted according to the Clinical and Laboratory Standards Institute document M100-S14 (2014). Multidrug resistance was defined as non-susceptibility to at least three of the following antimicrobials or antimicrobial classes: aminoglycosides, macrolides, clindamycin, fluoroquinolones, trimethoprim-sulfamethoxazole, tetracycline, and mupirocin.[204]

*DNA sequencing and analyses:*

DNA extraction was conducted on the MagNA Pure 96 Instrument for automated DNA extraction using the Pathogen Universal 200 protocol (Roche Life Science). The quality of the extracted DNA was assessed with the Epoch Microplate Spectrophotometer (Biotek, Winooski, VT) and ran on an automated capillary electrophoresis system (Qiaxcel Advanced System; Qiagen, Valencia, CA). Genomic DNA was quantified using the high-sensitivity double stranded DNA assay on the Qubit 2.0 fluorometer (Invitrogen, Life Technologies, Grand Island, NY). Sequencing libraries were prepared using the NexteraXT DNA sample preparation kit (Illumina Inc., San Diego, CA) following manufacturer's instructions. DNA libraries were sequenced on a MiSeq sequencing instrument (Illumina Inc., San Diego, CA) following manufacturer's instructions.

Sequencing data were imported and analyzed using CLC Genomics Workbench 8.1 (Qiagen, Redwood City, CA). Paired-end reads were mapped to a reference strain FPR3757 (accession NC_007793). FPR3757 was the first USA300, ST-8 strain to be completely sequenced; this strain was recovered from a skin abscess, and serves as a good reference strain for our comparative genome analysis.[102] The Fixed Ploidy Variant Detection tool was used to identify single nucleotide polymorphisms (SNPs) and Indels and Structural Variants tool was used to identify insertions and deletions. Local realignment was performed to improve mapping in areas around insertions and deletions using an algorithm described by Homer et al.[205] SNPs were included in analyses if that position contained at least 12 high-quality reads and 90% or more of them supported an alternate allele different from the FPR3757 reference.

For comparative genome evaluation, SNPs were used as a measure of genetic pairwise distances between strains. A SNP matrix was generated using the Reference Sequence Alignment based Phylogeny builder (REALPHY: http://realphy.unibas.ch/fcgi/realphy). The output consisted of 53,840 positions per genome. The SNP matrix was uploaded onto MEGA7 and CLCGenomics Workbench 8.5.1 for phylogenetic analyses. Phylogeny was inferred using neighbor joining and the maximum likelihood method based on the Jukes-Cantor model with 500 bootstrap replicates. Consistent with previous literature, clusters of strains were indicated by bootstrap values of >70% for maximum likelihood and neighbor joining analyses.[74]

Multilocus sequence typing (MLST) was determined from the sequence data by extracting the sequence at the specific loci of the seven housekeeping genes using the CLCGenomics Workbench 8.5.1 and the *S. aureus* MLST scheme (www.pubmlst.org; downloaded November 2015). Isolates were considered to be USA300 if they were ST8 and the PVL genes, as previously validated.[21,206]

Mobile genetic elements (MGEs) were detected *in silico*. Sequence reads were assembled *de novo* into contigs and were used to determine the presence and absence of MGEs. The assembled contigs were screened with PlasmidFinder to identify circular and integrated plasmids using a subset of replicon sequences from 139 fully sequenced plasmids associated with *S. aureus.*[207]

*Resistome:*

To assemble the resistome, the presence and absence of known antimicrobial resistance genes were identified using the ARG-ANNOT (Antibiotic Resistance Gene-ANNOTation) database followed by manual searching for chromosomal genes with amino acid variants.[208] The E-value cutoff was set at 1 $e^{-100}$. The genetic elements and accession numbers associated with susceptibility among the *S. aureus* isolates are listed in Table 5.1 and 5.2. In cases where multiple genes map at the same location of the assembly, the best match gene based on percentage identity was retained.

**Table 5.1** Genes associated with *S. aureus* antimicrobial resistance

| Antibiotic | Gene | Resistance Mechanism | Accession No. |
|---|---|---|---|
| **Erythromycin** | *msr(A)*<br>*mph(C)* | Erythromycin resistance efflux protein (macrolide and streptogramin B resistant phenotype)<br>Phosphotransferase inactivates macrolide antibiotic | CP003194<br>AF167161 |
| **Erythromycin and Clindamycin** | *ermC*<br>*ermA*<br>*ermB*<br>*ermT* | rRNA adenin N-6-methyltransferase<br>rRNA adenin N-6-methyltransferase<br>rRNA adenin N-6-methyltransferase<br>23S rRNA methylase | HE579068<br>BA00018.3<br>AB699882.1<br>HF583292 |
| **Gentamicin** | *aacA-aphD* | 6'-aminoglycoside N-acetyltransferase/ 2"-aminoglycoside phosphotransferase | FN433596.1 |
| **Penicillin** | *blaZ* | Class A beta-lactamase | BX571856.1 |
| **Methicillin** | mecA | Low-affinity PBP2 | BX571856.1 |
| **Tetracycline** | *tet(38)*<br>*tet(K)*<br>*tet(L)*<br>*tet(M)* | Putative transport system protein<br>MFS tetracycline efflux pump<br>MFS tetracycline efflux pump<br>Ribosomal protection protein | FN433596<br>FN433596<br>HF583292<br>CP002643 |
| **Trimethoprim** | *dfrA*<br>*dfrC*<br>*dfrG* | Trimethoprim-sensitive dihydrofolate reductase (insensitive dihdrofolate reductase) | CP002120 |
| **Vancomycin** | vanA | Low affinity peptidoglycan precursor | AE017171.1 |

**Table 5.2** Chromosomal genes with amino acid variants

| Antibiotic | Gene | Amino Acid Substitution | Accession No. |
|---|---|---|---|
| **Ciprofloxacin** | *gyrA*<br>*grlA*<br><br>*grlB* | S84L, E88K, G106D, S85P, E88G, E88L<br>S80F, S80Y, E84K, E84G, E84V, D432G, Y83N, A116E, I45M, A48T, D79V, V41G, S108N<br>R470D, E422D, P451S, P585S, D443E, R444S | BX571857.1 |
| **Rifampin** | *rpoB* | A473T, A477D, A477T, A477V, D471G, D471Y, D550G, H481D, H481N, H481Y, I527F, I527L, I527M, ins 475H, ins G475, L466S, M470T, N474K, Q456K, Q468K, Q468L, Q468R, Q565R, R484H, S463P, S464P, S486L, S529L | BX571857 |

*Discrepancy investigation:*

Discrepancies were defined as discordance between susceptibility results using Vitek 2 and predicted genotypic susceptibility. Discrepancies were investigated using gradient diffusion testing (Etest, bioMerieux, Durham, NC). Concordance was defined as agreement between susceptibility results from Vitek 2 or gradient diffusion with genotype. The Food and Drug Administration (FDA) guidelines on antimicrobial susceptibility devices were used to calculate rates of major and very major errors for genotypic susceptibility predictions for each antimicrobial tested. Major errors (ME) occur when the phenotypic result (Vitek 2) is susceptible and the genotypic prediction is resistant. Very major errors (VME) occur when the phenotypic result (Vitek 2) is resistant but the genotypic prediction is susceptible. The sensitivity, specificity, positive predictive value, and negative predictive values of the WGS genotypic prediction were determined. Table 5.3 lists the equations used.

**Table 5.3** Equations for calculation of ME, VME, sensitivity and specificity

A. Phenotype and genotype 2x2 table

| | | **Antimicrobial Susceptibility Phenotype** | |
|---|---|---|---|
| | | **Resistant** | **Susceptible** |
| **Genotype** | **Resistant** | **a** | **b** |
| | **Susceptible** | **c** | **d** |

B. Equations

| **Outcome** | **Equation** |
|---|---|
| ME | $\frac{a}{a+b+c+d}$ |
| VME | $\frac{c}{a+b+c+d}$ |
| Sensitivity | $\frac{a}{a+c}$ |
| Specificity | $\frac{d}{b+d}$ |
| PPV | $\frac{a}{a+b}$ |
| NPV | $\frac{d}{c+d}$ |

ME=major errors; VME=very major errors; PPV=positive predictive value; NPV=negative predictive value

*Statistical Analyses:*

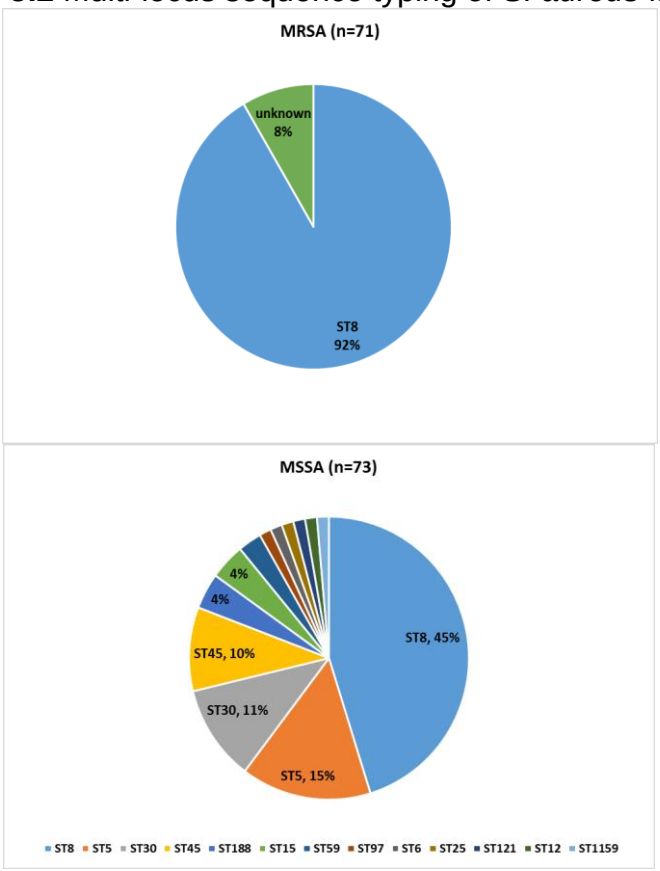
Statistical analyses were performed using SPSS 23.0® (IBM Corp, Armonk, NY, USA). Chi-square test or Fisher's Exact test was used for dichotomous or categorical variables. Student's t test or Wilcoxon rank sum test was used for continuous variables. The institutional review boards at UT Health Science Center San Antonio and University Health Systems approved the study.

## *Results*

*Multilocus sequencing typing of S. aureus*

We sequenced 144 *S. aureus* isolates (112 from SSTIs and 32 from nasal colonization) recovered from 144 patients presenting to a STARNet primary care clinic. Seventy-one strains were MRSA and 73 were MSSA. All MRSA isolates belonged to the ST8 clonal group (Figure 5.2). The majority (45%) of MSSA also belonged to ST8. Other MSSA strain types included: ST5, ST6, ST12, ST15, ST25, ST30, ST45, ST59, ST97, ST121, ST188, and ST1159. Four isolates had undefined MLST designations.

**Figure 5.2** Multi-locus sequence typing of *S. aureus* isolates



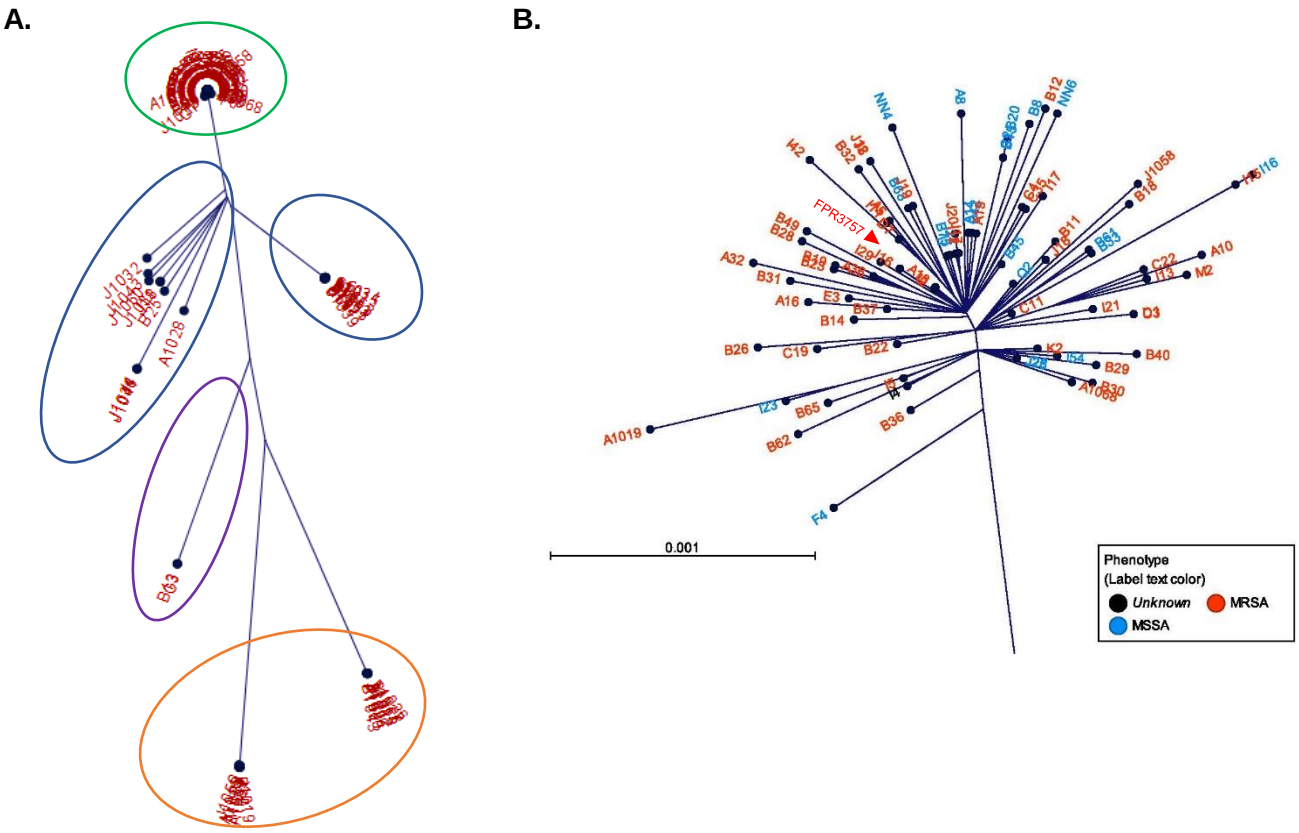

MRSA=methicillin resistant *S. aureus;* MSSA=methicillin susceptible *S. aureus*

*SNP-based phylogeny of S. aureus in South Texas*

To estimate the population structure, sample reads were mapped onto a single core reference genome, FPR3757. One-hundred and twenty three isolates had sufficient coverage for the analyses. We identified a total of 53,840 SNP sites compared to the reference sequence FPR3757. After excluding MGEs, the strains differed by an average of 3,548 SNPs (range 15 to 47,574). Based on the neighbor joining method, the isolates fell into 4 general groupings (Figure 5.3). The first group comprised only of ST8 isolates that grouped very tightly with FPR3757, differing by an average of 160 SNPs (range 15-1,215). Approximately 70% of the isolates clustered in Group 1 including a number of MSSA ST8 isolates. Group 2 encompassed two branches that were both similarly distant to the reference strain: one group comprised of mainly ST5 strains, which are known to be predominantly HA-MRSA strains, the other group was more heterogeneous without a common clone type. The third group comprised of a few strains that were found to be closely related to ST59. The fourth group comprised mainly of ST45 and ST30 MSSA strains.

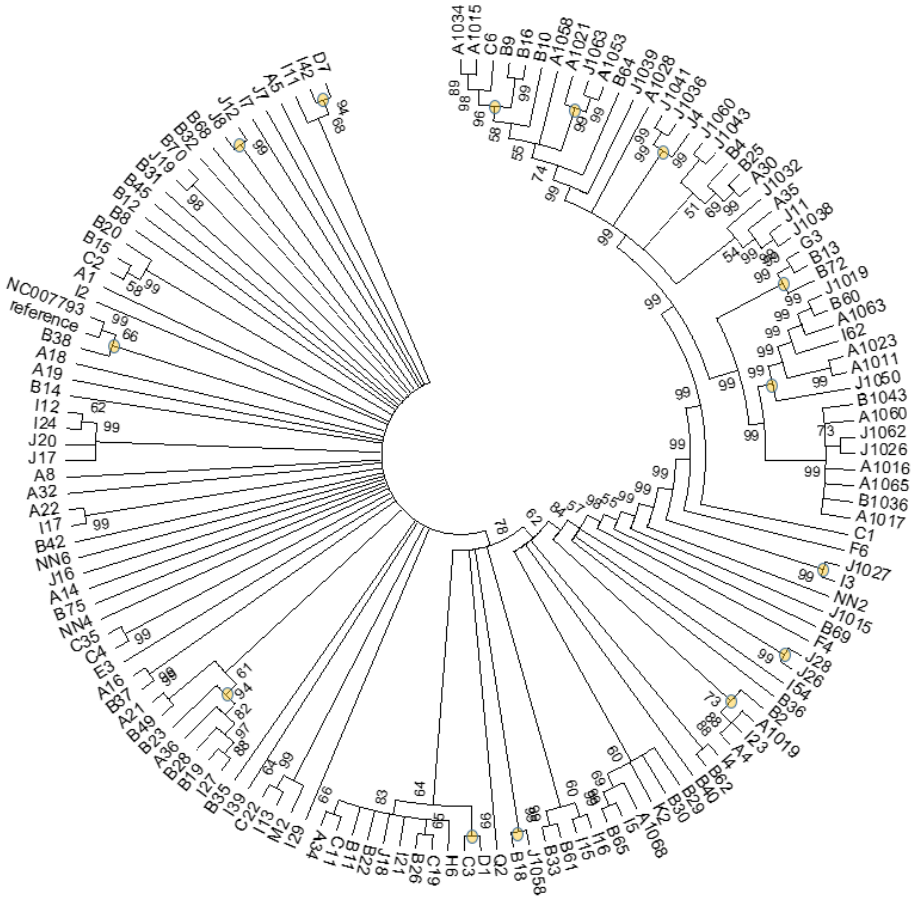
To provide another validation of the population structure, an approximate maximum likelihood tree was generated based on the similar alignment (Figure 5.4). With 500 resamples, approximately 65% of the branches had over 0.50 bootstrap supports. There were some secondary clusters (bootstrap support >0.70 and congruent with neighbor joining tree) that were mostly clonal, and were separated mostly MLST clonal types.

**Figure 5.3** SNP-based phylogenetic analyses of *S. aureus* strains




SNP based phylogenetic analysis. (A) Radial phylogenetic tree mapping the isolates based on the concatenated SNP distances. Green ring (group 1) primarily ST8 clustered tightly with reference FPR3757. Blue rings (group 2) comprised of two groups similarly distant to FPR3757. Purple ring (group 3) comprised of 3 ST59 isolates. Orange ring (group 4) comprised of ST30 and ST45 isolates. (B) Zoomed-in view of group 1 cluster comprised of ST8 isolates.

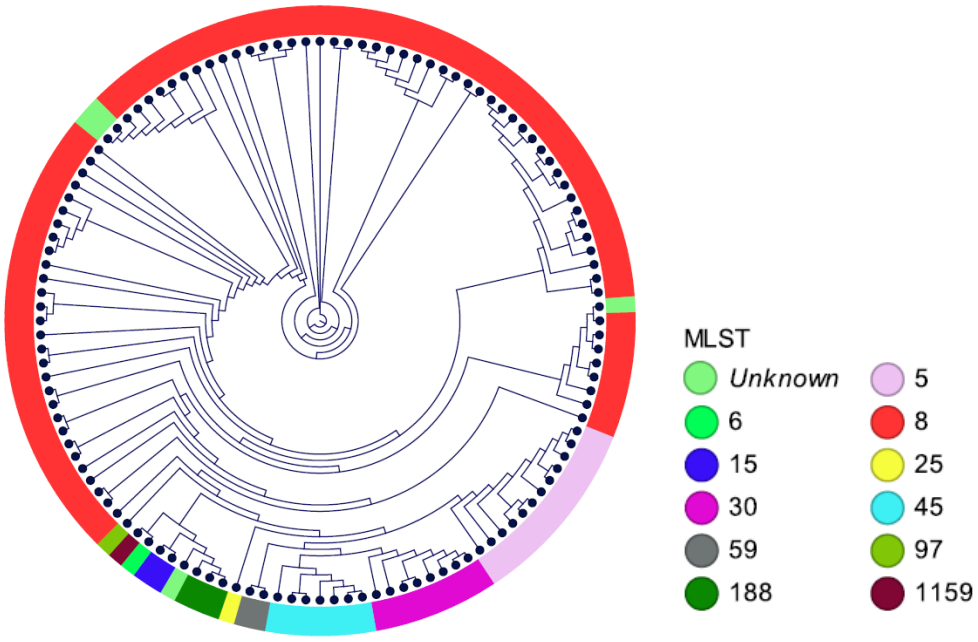
**Figure 5.4** Maximum-likelihood phylogenetic tree of *S. aureus* strains




Nodes supported by maximum-likelihood and neighbor joining bootstrap analysis are indicated by the yellow colored circles. Numbers at each node represent bootstrap values.

To evaluate the concordance of traditional MLST typing and WGS data, the MLST strain types were mapped onto the WGS phylogenetic tree (Figure 5.5). In this collection, isolates with the same MLST type predictably clustered into the same branch. Although isolates sharing the same ST cluster together, the high resolution of WGS demonstrated that the distance between individual isolates within each cluster varied from relatively close to more distant. This may suggest that the close clusters represent successful lineages that have undergone recent clonal expansion, whereas the distantly-related clades may represent rarer, less successful lineages.

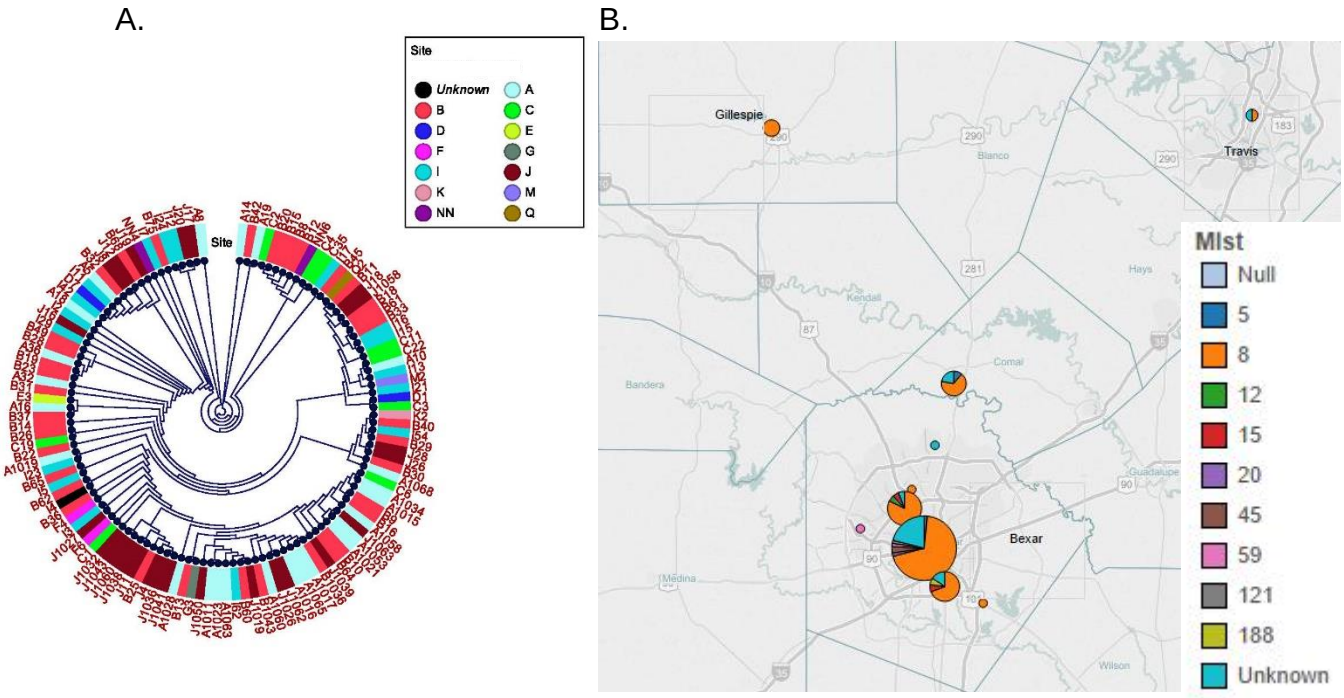
**Figure 5.5** SNP-based phylogenetic analysis and MLST




Multilocus strain types were computationally mapped onto the phylogenetic tree. The colored outer ring denotes the MLST type. MLST=multilocus sequence type.

To identify whether particular strains clustered together by geographic region, the location of the practice site was mapped onto the WGS phylogenetic tree. The MLST and phylogenetic analysis revealed that specific clones were not necessarily specific to particular geographic locations (Figure 5.6), with the exception that all *S. aureus* strains from Bulverde, TX were ST59.

**Figure 5.6** Geographic epidemiology of community-associated *S. aureus* strains in South Texas




(A) Phylogenetic cladogram with the outer colors coded by the location of the clinics. (B) Proportion of multi-locus sequence types by zip code.

*Antimicrobial resistance determinants of S. aureus:*

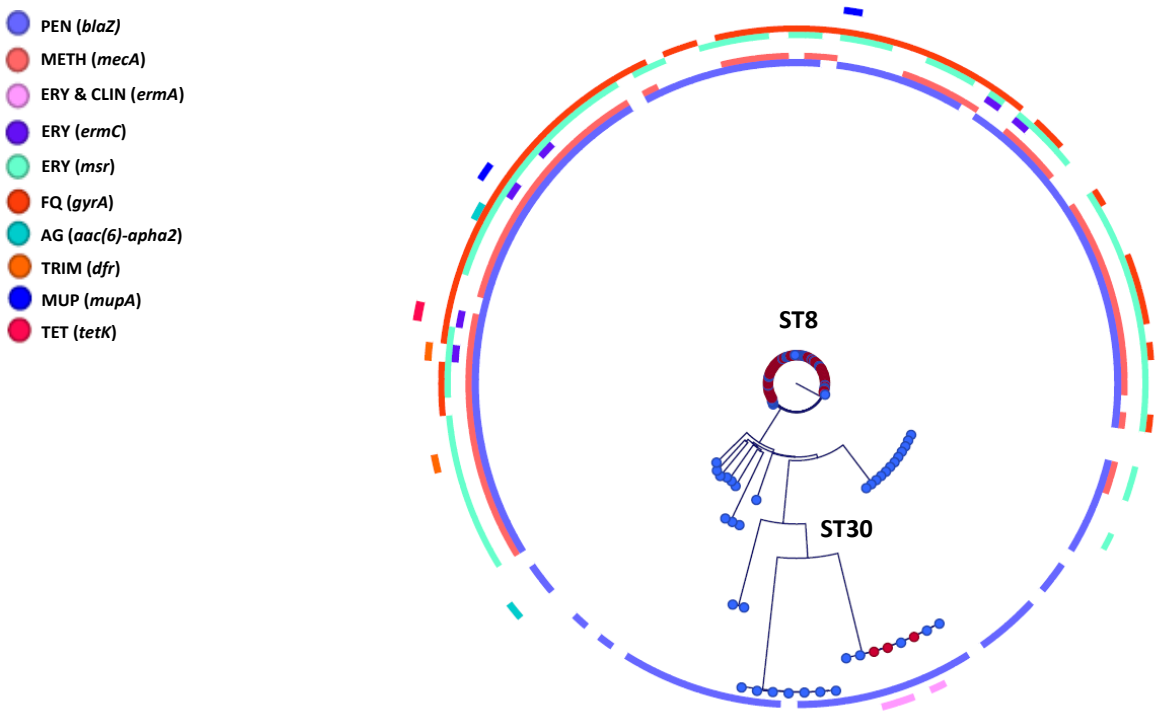
The antibiograms of one hundred and forty three isolates (32 nasal isolates and 111 SSTI isolates) were analyzed. None of the strains were resistant to vancomycin, linezolid, daptomycin, or rifampin. Table 5.4 displays the concordance of phenotype and genotype. Genetic determinants were specifically isolated within specific clades on the phylogenetic tree (Figure 5.7). Notably, the presence of drug resistance differed substantially between ST8 isolates and the remainder of the sample, with methicillin resistance having the most marked difference. The presence of resistance determinants for aminoglycosides, tetracycline, mupirocin, and trimethoprim occurred infrequently and primarily only among ST8 isolates.

**Table 5.4** Comparison of whole genome detection of antimicrobial resistance determinants and phenotype

| | Phenotype: susceptible | | | Phenotype: resistant | | | Error Rate (%) | | Sensitivity | Specificity | PPV | NPV |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Genotype | | | Genotype | | | | | | | |
| Antibiotic | # S | S | R | # R | S | R | VME | ME | | | | |
| Ciprofloxacin | 87 | 78 | 7 | 56 | 3 | 53 | 2.1 | 4.9 | 95 | 90 | 85 | 96 |
| Clindamycin | 133 | 133 | 0 | 10 | 0 | 10 | 0 | 0 | 100 | 100 | 100 | 100 |
| Erythromycin | 70 | 62 | 8 | 73 | 3 | 70 | 2.1 | 5.6 | 96 | 88 | 90 | 94 |
| Gentamicin | 141 | 141 | 0 | 2 | 0 | 2 | 0 | 0 | 100 | 100 | 100 | 100 |
| Mupirocin | 140 | 140 | 0 | 3 | 1 | 2 | 0.7 | 0 | 67 | 100 | 100 | 99 |
| Oxacillin | 71 | 67 | 4 | 72 | 2 | 70 | 1.4 | 2.8 | 97 | 93 | 93 | 97 |
| Rifampin | 143 | 143 | 0 | 0 | 0 | 0 | 0 | 0 | n/a | 100 | n/a | 100 |
| Tetracycline | 141 | 141 | 0 | 2 | 0 | 2 | 0 | 0 | 100 | 100 | 100 | 100 |
| Trimethoprim | 139 | 139 | 0 | 4 | 1 | 3 | 0.7 | 0 | 75 | 100 | 100 | 99 |
| Vancomycin | 143 | 143 | 0 | 0 | 0 | 0 | 0 | 0 | n/a | 100 | n/a | 100 |

S=susceptible; R=resistant; VME=very major errors; ME=major errors; PPV=positive predictive value, NPV=negative predictive value. Sensitivity, specificity, PPV, and NPV are shown as percentages.

**Figure 5.7** Distribution of antimicrobial resistance determinants




Phylogeny of *S. aureus* strains based on concatenated SNP distances from reference genome FPR3757. Nodes are marked in red for multidrug-resistant isolates (phenotypically resistant to 1 antibiotic in $\geq$ 3 classes). The presence of drug resistance determinants are indicated in concentric rings around the phylogenetic tree. The two clades with multidrug-resistant *S. aureus* in South Texas were among ST8 and ST30.

*Epidemiological features of multidrug resistance*

Approximately 31% (45) of the isolates were multidrug-resistant. Most multidrug-resistant isolates were ST8 (91%); one isolate was an ST30, one isolate was ST121, and 2 with unknown ST designations (Figure 5.6). Approximately 42% of ST8 strains were multidrug-resistant. A higher proportion of SSTI isolates were multidrug-resistant isolates compared to colonizing isolates (91% vs 9%; $p<0.05$). Notably, there was no significant difference in prior antibiotic exposures between cases with multidrug-resistant isolates compared to non-multidrug-resistant isolates (17% vs. 14%; $p=0.60$) (Table 5.5). Other features associated with multidrug-resistant isolates included African American race ($p<0.01$) and history of prior skin infections ($p=0.05$).

**Table 5.5** Characteristics of patients with multidrug-resistant compared to non-multidrug-resistant community-associated *S. aureus* strains

| Characteristic | Overall n = 143 | MDR n = 45 | No MDR n = 98 | *p* |
|---|---|---|---|---|
| Mean age, yrs. (±SD) | 41 (±14) | 43 (±12) | 41 (±13) | 0.46 |
| Gender | | | | |
| Male | 72 (50%) | 25 (56) | 47 (48) | 0.39 |
| Race/Ethnicity | | | | |
| African American | 8 (6) | 6 (13) | 2 (2) | 0.01* |
| Hispanic | 111 (78) | 34 (76) | 77 (79) | 0.67 |
| Diabetes | 40 (28) | 14 (31) | 26 (27) | 0.57 |
| Obese (BMI ≥ 30) | 75 (56) | 23 (53) | 52 (58) | 0.64 |
| Peripheral vascular disease | 0 | 0 | 0 | n/a |
| Chronic non-infectious skin disorder | 1 (1) | 0 (0) | 1 (2) | 1.00 |
| HIV | 0 | 0 | 0 | n/a |
| Cancer | 0 | 0 | 0 | n/a |
| Chemotherapy | 0 | 0 | 0 | n/a |
| Provides Healthcare to Others | 5 (4) | 1 (2) | 4 (4) | 1.00 |
| MRSA Phenotype | 72 (50) | 33 (73) | 40 (41) | <0.01* |
| Site – SSTI (vs nasal colonization) | 111 (78) | 41 (91) | 70 (71) | <0.01* |
| Prior SSTI | 31 (22) | 12 (27) | 19 (19) | 0.33 |
| Prior antibiotic history | 19 (13) | 5 (11) | 14 (14) | 0.60 |
| Prior MRSA infections | 3 (3) | 3 (7) | 0 (0) | 0.05* |

MDR=multidrug-resistant; yrs.=years; SD=standard deviation; BMI=body mass index; MRSA=methicillin resistant *S. aureus*; SSTI=skin and soft tissue infection; (*) indicates statistical significance.

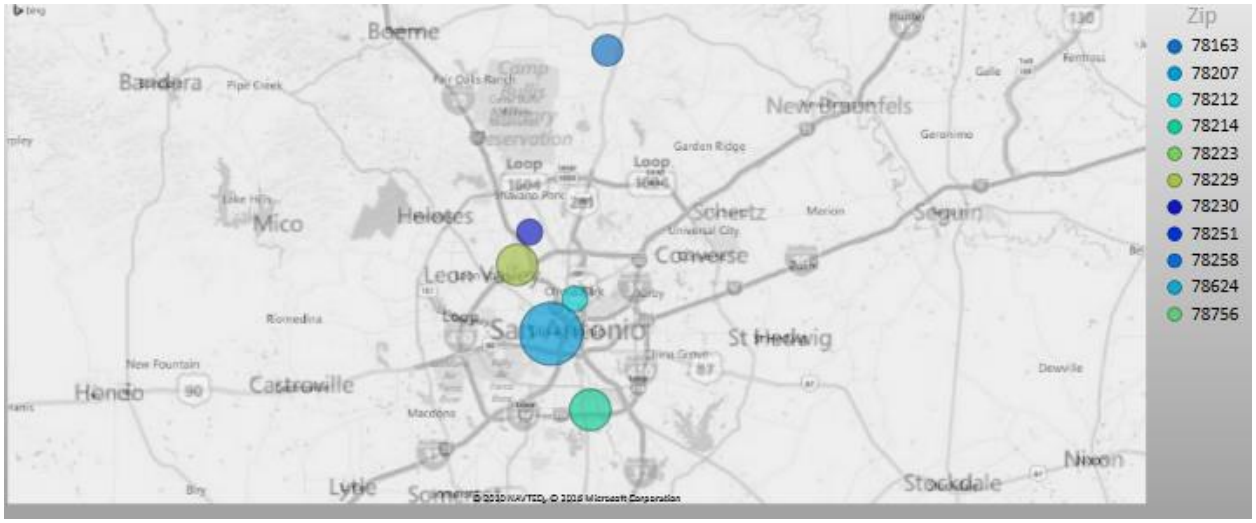
*Plasmid characterization*

On average, the isolates carried ~3 plasmids (range 0 to 6). The occurrence of pUSA300-like plasmids (e.g., pUSA02 and pUSA03) was rare and was only detected in 2 ST8 isolates. However, we identified other plasmids that were widespread. In the case for pSJH101 (*rep* 16), its *rep* genes were detected in strains belonging to different lineages (ST8, ST5, ST59, ST45, and ST30). This supports the notion of the transferring of MGEs and resistance genes among different *S. aureus* clonal lineages. Three MRSA isolates were resistant to 5 antimicrobial classes including β-lactam, fluoroquinolones, erythromycin, clindamycin, and tetracycline harboring plasmids resembling pSJH901, pSJH101, pKH14, pE5, SAP101A, and pMSSA476 (Appendix C). One MSSA ST121 isolate was found to be resistant to erythromycin, clindamycin, tetracycline, and trimethoprim-sulfamethoxazole harboring plasmids resembling pSJH901, pSJH101, and pMSSA476.

*Multidrug-resistant S. aureus by geographic region*

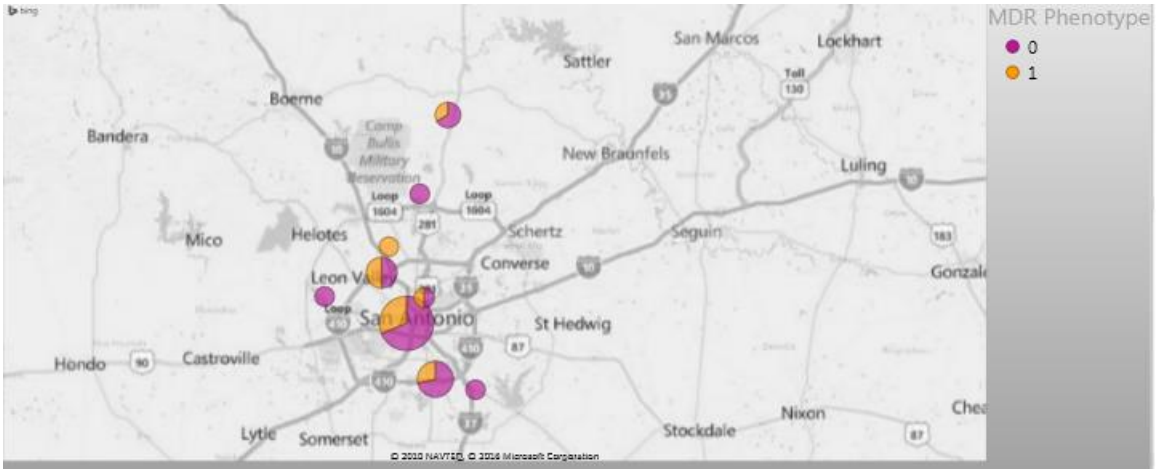
When evaluating geographic clustering, we found more than half of the multidrug-resistant isolates clustered within the Inner West Side of San Antonio, a predominantly Hispanic and African American community with household incomes significantly below state average (Figure 5.8). However, the rate of multidrug-resistant strains (among areas with $\geq$ 2 isolates) was disproportionately higher (50% of isolates in this geographic region were multidrug-resistant) in the Northwest side of San Antonio, the location of the South Texas Medical Center (Figure 5.9).

**Figure 5.8** Count of multidrug-resistant *S. aureus* strains by geographic region




Colors of the circles indicate zip codes that multidrug-resistant *S. aureus* strains were collected from and the size of the circles represent the count of multidrug-resistant strains.

**Figure 5.9** Proportion of multidrug-resistant *S. aureus* strains by geographic region




The size of the circles represent the count of *S. aureus* isolates from each region. Proportion of multi-drug resistance *S. aureus* strains in each region is depicted in colors: orange=multidrug resistant and pink=non-multidrug resistant strains.

*Resistome of community-associated S. aureus*

This section describes the distribution of antimicrobial resistance determinants as well as the extent the WGS data correlate with standard antimicrobial susceptibility testing.

*Oxacillin.* Seventy three isolates (49%) were identified as MRSA by initial phenotypic testing. The very major error rate was 1.4% and the major error rate was 2.8%. The sensitivity and specificity were 97% and 93%, respectively. The *mecA* gene was not detected in 2 isolates that were oxacillin resistant in initial phenotypic testing. On repeat testing, both isolates were susceptible to oxacillin (Table 5.6). The *mecA* gene was detected in 4 oxacillin-susceptible isolates in initial phenotypic testing. Two of these isolates tested resistant while 2 isolates remained susceptible upon repeat testing. Isolates harboring the *mecA* gene were ST-8 (70) and unknown (4).

*Ciprofloxacin.* Fifty six isolates (39%) were ciprofloxacin resistant by initial phenotypic testing. The very major error rate was 2.1% (3/143) and the major error rate was 4.9% (7/143). The sensitivity and specificity were 95% and 90%, respectively. All 3 very major errors were susceptible on repeat testing. Four of the 7 major errors were resistant on repeat testing. Fifty-three of the isolates that predicted to be resistant by genotype had the amino acid substitution S84L in *gyrA,*

and were only found among USA300 strains. This finding is explored in more detail in the following section.

*Erythromycin and Clindamycin.* Seventy three isolates (52%) were erythromycin resistant by initial phenotypic testing. The very major error rate was 2.1% (3/143) and major error was 5.6% (8/143). The sensitivity and specificity were 96% and 89%, respectively. Upon repeat testing, all 3 very major errors and 8 major errors aligned with the genotype. Of the 70 isolates that were confirmed erythromycin resistant, 10 isolates were clindamycin resistant. Of these isolates, 4 had inducible resistance by D-test and contained only the *ermA.* The 6 clindamycin resistant isolates without inducible resistance contained both the *ermC* and *msrA* genes. The 63 isolates that were resistant to erythromycin but susceptible to clindamycin only contained the *msrA* gene. There were no errors observed for clindamycin. Isolates harboring *ermC* and *msr* were only distributed among ST-8 strains, while *ermA* was primarily found in ST-30 strains.

*Gentamicin.* Two ST-8 strains (1 MRSA and 1 MSSA) were gentamicin resistant by phenotypic testing. There were no errors observed based on the presence or absence of *aacA(6')-aphD(2')*. The presence or absence of other putative aminoglycoside resistance determinants including *aadD, ant(6)-Ia*, *aph(3')-III,* and *spc* were not correlated with gentamicin resistance in this cohort.

*Tetracycline.* Two isolates were tetracycline resistant by phenotypic testing. There were no errors observed based on the presence or absence of *tetK*. Both tetracycline-resistant strains had an elevated doxycycline MIC of 4 mg/L (considered phenotypically resistant according to EUCAST). The presence or absence of the efflux gene *tet38* was not correlated with tetracycline resistance in this collection.

*Vancomycin.* Vancomycin resistance was not identified in this collection of isolates by either phenotypic or genotypic testing. While the specificity was 100%, the sensitivity could not be estimated due to the lack of *vanA* among the isolates.

*Trimethoprim.* Four isolates (2.8%) were resistant to trimethoprim by phenotypic testing. The very major error rate was 0.7% and no major errors were detected. The sensitivity and specificity were 75% and 100%, respectively. The one very major error tested susceptible upon repeat testing.

*Mupirocin.* Two isolates (2.1%) displayed mupirocin resistance by initial phenotypic testing. One isolate harbored the *mupA* gene and displayed high level mupirocin resistance (MIC≥516 µg/mL). One isolate displayed low-level mupirocin resistance (MIC=32 µg/mL) and contained a mutation in the native *ileS* gene.

**Table 5.6** Discrepancy testing of whole genome detection of antimicrobial resistance determinants and phenotype

| ***Very Major Errors*** | **Isolate** | **Initial Phenotype** | **Repeat Phenotype** | **Genotype** | **Resolved** |
|---|---|---|---|---|---|
| Cipro | I5 | R | 0.125 S | No *gyrA or grlA* mutation detected | Y |
| | I23 | R | 0.94 S | No *gyrA or grlA* mutation detected | Y |
| | I39 | R | 0.125 S | No *gyrA or grlA* mutation detected | Y |
| Erythromycin | B23 | R | 0.125 S | No *erm* or *msrA* gene detected | Y |
| | I7 | R | 0.125 S | No *erm* or *msrA* gene detected | Y |
| | I17 | R | 0.125 S | No *erm* or *msrA* gene detected | Y |
| Oxacillin | B23 | Oxacillin MIC>4 (R) Cefoxitin Screen (+) | 0.5 S | No *mecA* gene detected | Y |
| | I7 | Oxacillin MIC>4 (R) Cefoxitin Screen (+) | 0.25 S | No *mecA* gene detected | Y |
| Trimethoprim | B23 | R | 0.04 S | *No dfrA, dfrG, or dfrB mutation detected* | Y |
| ***Major Errors*** | | | | | |
| Cipro | A8 | S | $\geq$ 32 R | *gyrA* mutation detected | Y |
| | I7 | S | 8 R | *gyrA* mutation detected | Y |
| | I17 | S | 6 R | *gyrA* mutation detected | Y |
| | I42 | S | 16 R | *gyrA* mutation detected | Y |
| Erythromycin | B30 | S | 32 R | *msr* gene detected | Y |
| | C11 | S | 32 R | *msr* gene detected | Y |
| | I5 | S | 48 R | *msr* gene detected | Y |
| | I11 | S | 24 R | *msr* gene detected | Y |
| | I21 | S | 64 R | *msr* gene detected | Y |
| | I23 | S | 64 R | *msr* gene detected | Y |
| | I24 | S | 48 R | *msr* gene detected | Y |
| | J17 | S | 32 R | *msr* gene detected | Y |

**Table 5.6** Discrepancy testing of whole genome detection of antimicrobial resistance determinants and phenotype (Continued)

| | | | | | |
|---|---|---|---|---|---|
| Oxacillin | A8 | Oxacillin MIC<0.25 (S) Cefoxitin Screen (-) | 64 R sub colonies | *mecA* gene detected | Y |
| | C11 | Oxacillin MIC=1 (S) Cefoxitin Screen (+) | 1 S | *mecA* gene detected | N |
| | I5 | Oxacillin MIC<0.25 (S) Cefoxitin Screen (-) | 1 S | *mecA* gene detected | N |
| | I11 | Oxacillin MIC=0.5 (S) Cefoxitin Screen (-) | 32 R sub colonies | *mecA* gene detected | Y |

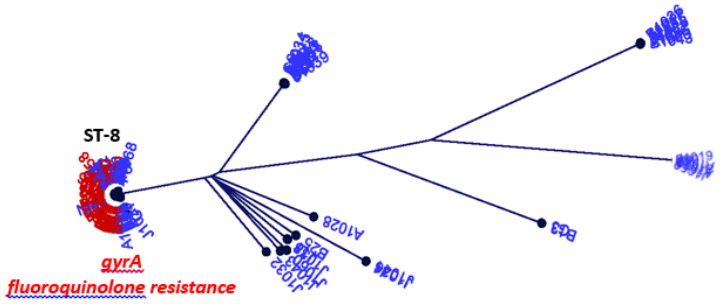
MIC=minimum inhibitory concentration

*Fluoroquinolone resistance among USA300*

To explore the association of role of antimicrobial resistance determinants in the evolution of *S. aureus*, the presence and absence of specific resistance mechanisms were mapped onto the WGS phylogenetic tree. The phylogeny of *S. aureus* strains revealed that strains containing the mutation that confers fluoroquinolone resistance within the *gyrA* (leu84ser) gene clustered among USA300 strains in the ST8 cluster (Figure 5.10a). When evaluating the population structure of USA300 strains, the strains broadly clustered within two major clades based on the presence of fluoroquinolone resistance (Figure 5.10b). This is consistent with prior studies suggesting that fluoroquinolones might have further promoted the clonal expansion of USA300.[74,209] However, whether the fluoroquinolone resistant strains are replacing the non-resistant types remains yet to be investigated.

**Figure 5.10** Fluoroquinolone resistance among *S. aureus*

**A.**




**B.**

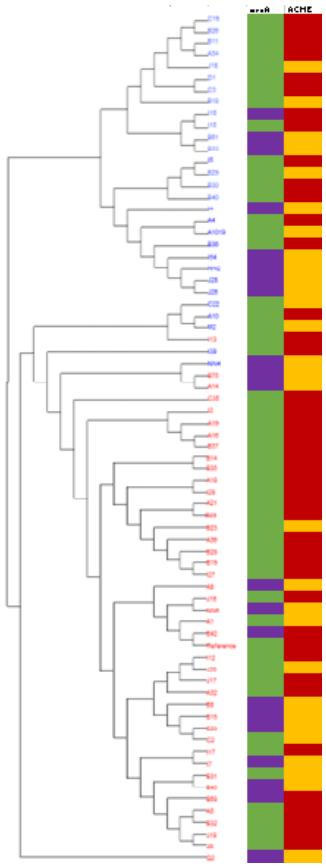


**Figure 5.10** Fluoroquinolone resistance among *S. aureus* (continued)

**A.** Radial tree of the chromosomal mutation *gyrA* conferring fluoroquinolone resistance (presence of chromosomal mutation marked in red). **B.** Maximum likelihood tree of USA300 *S. aureus* strains. Strains with the presence of chromosomal mutation marked in red and absence in blue. Adjacent to the tree is a heatmap of the presence of the *mecA* gene (presence=green; absence=purple) and the arginine catabolic mobile element (presence=red; absence=orange).

## Discussion

The emergence of multidrug-resistant *S. aureus* strains in outpatients could complicate disease management and contribute to development of persistent or recurrent community-associated MRSA infections. While multidrug resistance has been reported in different regions in the U.S., the magnitude of resistance among community-associated *S. aureus* infections in South Texas were unknown.

This study identified that multidrug resistance among community-associated *S. aureus* strains has emerged in South Texas. Approximately one-third of *S. aureus* isolates were multidrug-resistant. The majority of the multidrug-resistant strains were USA300 (ST8, PVL-positive). This study suggests that the USA300 lineage has potential to overcome the fitness cost of multidrug resistance.[93] Approximately 10% of the isolates were carrying resistance mechanisms for trimethoprim-sulfamethoxazole, clindamycin, or tetracyclines, which are considered first line treatments against potential CA-MRSA SSTIs. Currently, culture and antimicrobial susceptibility testing for presumed *S. aureus* SSTIs is infrequently practiced in the outpatient setting. Our findings underscore the growing importance of microbial culture and antimicrobial resistance detection practices in the outpatient setting to minimize risk for treatment failures and inappropriate antimicrobial exposures.

This investigation identified African American race and geographic areas of San Antonio associated with a higher proportion of multidrug resistance *S. aureus*. While several studies have reported that African American race is a risk factor for

MRSA infections,[24,210,211] this is the first study to observe that this population may also be at increased risk for acquiring multidrug-resistant *S. aureus* strains. Notably, there was no significant difference in prior antibiotic exposures between cases with multidrug-resistant isolates compared to non-multidrug-resistant isolates. This indicates that multidrug-resistant strains are likely being transmitted person to person or antimicrobial resistance determinants are being directly acquired from other bacteria. The density of multidrug-resistant *S. aureus* was geographically highest in the Northwest side of San Antonio, the location of the South Texas Medical Center. Consistent with other studies, this indicates the possibility of direct acquisition of resistance determinants from multidrug-resistant health care-associated MRSA strains.[22,43-45,118] Further studies are needed to determine the causes of these potential racial and geographical disparities, and the role hospitals play in the spread of multidrug-resistant *S. aureus* strains back into the community.

The phylogeny of *S. aureus* strains revealed that strains containing the mutation that confers fluoroquinolone resistance within the *gyrA* (leu84ser) clustered only among USA300 strains. When evaluating the population structure of USA300 strains, we identified the strains clustered within two major clades based on the presence of fluoroquinolone resistance. This is consistent with recent studies in various geographic regions in the U.S. Previous epidemiological studies have associated increased fluoroquinolone use with the rise of CA-MRSA incidence rates.[74,209] Because fluoroquinolones are not considered an appropriate

treatment option against *S. aureus* infections, this highlights the potential collateral damage of antibiotic use. The selective pressures of *gyrA* mutations among USA300 *S. aureus* strains requires further exploration.

This study described the predictive patterns of antimicrobial resistance determinants based on genetic mechanisms most commonly observed among community-associated *S. aureus* isolates. The final results demonstrated a high level of concordance. After considering the findings from discrepancy testing, the overall VME and ME were 0% and 1.4%, respectively. This is comparable with the error rates for current phenotypic methodologies including the Vitek 2 and the Phoenix automated microbiology systems.[212,213] Moreover, the error rates are within the acceptable limits set by the FDA for susceptibility testing methods (VME<1.5% and ME<3%).

Almost all of the initially observed discrepancies were resolved by repeat phenotypic testing. Interestingly, all VMEs were rectified with subsequent discrepancy testing that aligned to the genotype. This indicates the importance of potential lab errors that might contribute to observed phenotypic variation including, inoculum density, temperature conditions, contamination, media, labeling, and material storage. Most MEs were resolved after repeat phenotypic testing. The MEs were from the detection of oxacillin resistance. The *mecA* gene was detected while phenotypically displaying susceptibility to oxacillin (MIC $\leq$ 1 ug/mL). By definition, MRSA strains have an oxacillin MIC of ≥4 ug/mL or harbor the *mecA* gene.[214] However, the majority of *S. aureus* strains display

heteroresistance. Phenotypically oxacillin susceptible and *mecA* positive *S. aureus* strains have been increasingly reported.[215-217] The clinical significance and approach to these strains remain unclear. While some investigations have shown that these strains might be responsive to β-lactam, others have demonstrated that β-lactams might lead to inducible resistance during treatment leading to treatment failure.[218]

Over the last decade, significant advances have been made with next generation sequencing technology. Current sequence times have been reported to be as short ~24 hours with sequencing costs as low as $65 for an isolate.[181,182] As run times, sequencing costs, and high technical requirements continue to decline, WGS may be a promising tool for resistance prediction in *S. aureus.* However, there are great hurdles that need to be overcome before this technology can be applied. Despite the promising concordance of genotype and phenotype of antimicrobial resistance determinants in these studies, WGS will not be employed to replace AST for *S. aureus* in the near future for several reasons. First, WGS remains prohibitively expensive and may be less sensitive to culture or PCR when applied directly to clinical samples. Currently, phenotypic AST (e.g., automated broth-culture methods or disc diffusion) remains faster and cheaper. Second, there are major challenges for *in silico* resistance detection. When dealing with mixed samples, a technical barrier is in assigning which pathogen a plasmid-mediated resistance gene belongs. Furthermore, the presence of antimicrobial resistance determinants without phenotypic expression highlights the need for additional

gene expression data for select genes. Continued advancement in metagenomics and transcriptomics using single cell sequencing might help overcome these barriers. Importantly, to determine the reliability of these genotypic predictions in the clinical setting, particularly regarding novel resistance mechanisms, ongoing investigation and establishment of a central publicly available database is required. Several antimicrobial resistance databases exist including: Antibiotic Resistance Genes Database,[219] Resfinder,[188] and the Comprehensive Antibiotic Resistance Database.[220] However, these databases are neither exhaustive nor regularly updated, and do not allow the detection of point mutations in chromosomal target genes associated with resistance. Therefore, analyses using these tools are extremely time consuming and involve multiple layers of analyses. The challenge is to develop a robust tool that is easily accessible to clinicians without bioinformatics knowledge. One such potential platform in its beginning stages has been recently described.[193]

*Limitations:*

There were several limitations to this study. First, the epidemiological data were limited to reveal important transmission networks. The geographic associations were limited to the locations of the clinics that served the patients and not specific household addresses. In addition, this study is inherent to sampling bias from the participating clinics. The low or absent level of resistance to certain antibiotics in this collection prohibited the ability assess predictions.

# CHAPTER SIX

# Specific aim 2

## Genomic heterogeneity and prediction of *Staphylococcus aureus* skin and soft tissue infections

### *Introduction*

*S. aureus* is a major human pathogen and a global public health issue. It is considered an opportunistic pathogen as it asymptomatically colonizes its host but can occasionally cause diseases that range in severity from relatively minor skin and soft tissue infections (SSTI) to life-threatening cases of pneumonia and endocarditis.[37] *S. aureus* typically exists as a commensal and colonizer of human skin and mucosa. Approximately 12-30% of individuals are persistent *S. aureus* carriers and 30% are intermittent carriers of *S. aureus*.[163,167,221] Nasal colonization by *S. aureus* has been established as one of the most important risk factors for invasive infections.[72,160,164,168,177,197,222-224] In a prospective study, Ellis et al. found a higher risk (relative risk 3.1, 95% CI 1.5-6.5) for nasal carriers of MRSA to acquire a CA-MRSA infection (i.e., cellulitis, abscess).[172] Recent studies have identified that 70-90% of the colonized strain belonged to the same *S. aureus* clonal type as the strain involved in the skin infection.[91,225] However, the pathogenetic mechanisms of why certain strains remain sole colonizers while some go on to produce SSTIs are unclear.

The advances of WGS techniques have offered new insights into *S. aureus* diversification during asymptomatic carriage and disease. Previous studies have suggested that the intra-host evolution from asymptomatic carriage to subsequent bloodstream infection may be driven by minimal genetic differences to discriminate pathogenic strains and sole colonizing strains.[197] However, the genetic differences in pathogens during carriage and SSTIs have yet to be clearly established. In addition, host features and clinical context are likely to be important factors that have not yet been studied in conjunction with genomic investigations. Studies to better understand the ecology, pathogenesis, and epidemiology of *S. aureus* nasal carriage and its comparison to skin infections are needed to develop targeted preventative measures.

The identification of specific variants that might be associated with *S. aureus* pathogenicity can be performed using a technique called genome-wide association study (GWAS). GWAS has been broadly used in human genetics to identify single nucleotide polymorphisms (SNPs) associated with complex diseases, ranging from cancer to mental health.[226,227] This procedure compares SNPs across a population including individuals with and without the disease and aims to report SNPs enriched in the disease cases but absent in the healthy controls as potential risk factors. Bacterial GWAS allows researchers to link individual elements of the genotype including core genes, mobile genetic elements, and SNPs, to specific phenotypes.[228-230] While this technique among bacteria remains relatively novel, it has been successfully performed using

*Campylobacter*,[231] *S. aureus*,[184,200] and *Streptococcus pneumoniae*.[232] This approach gives an opportunity to investigate the molecular mechanisms of SSTI pathogenicity down to single nucleotide changes. Ultimately, this high resolution insight into pathogenicity mechanisms might contribute to the foundation for future applications of genome sequencing in predicting the severity of *S. aureus* infections in clinical settings and surveillance studies.

Herein we sought to 1) describe the epidemiologic factors associated with *S. aureus* SSTIs and nasal colonization in the South Texas primary care setting, 2) use whole genome sequences to describe the diversity and distribution of virulence mechanisms among community-associated *S. aureus* isolates, 3) conduct a bacterial GWAS to identify *S. aureus* genetic signatures associated with SSTI isolates compared to colonization isolates, and 4) derive a predictive model for SSTI.

***Methods:***

*Study setting and population:*

We performed this investigation among a well-described cohort of patients with SSTIs in the primary care setting. Details of this cohort have been described previously.[49] Briefly, this study was conducted in collaboration with fourteen clinics within the South Texas Ambulatory Research Network (STARNet), a practice-based research network composed of 108 urban, suburban, and rural primary care clinics distributed throughout the South Texas region, from 2007 to 2015. Patients were eligible for study enrollment if they provided informed consent, were 18 years of age or older, and presented to one of the participating clinics with an SSTI. Healthcare providers collected wounds sample and patient information from each patient (e.g., demographics, infection characteristics, clinical information). Patients who presented to these clinics without an SSTI were assessed for colonization and were surveyed for demographic and clinical information. This study was reviewed and approved by the Institutional Review Board of UT Health Science Center at San Antonio and University Health Systems, San Antonio.

*Bacterial isolates*:

We selected 112 isolates of community-associated *S. aureus* from the wounds of patients presenting with SSTIs and 32 *S. aureus* nasal colonization isolates from patients without SSTIs for whole genome sequencing.

*Microbiological analysis:*

Samples were plated onto pre-filled blood agar plates and incubated at 35°C to 37°C for 24 hours, then sub-cultured to MRSA selective agar (MRSASelect chromogenic agar plates; Bio-Rad Laboratories, Hercules, CA). Latex agglutination tests (StaphAurex®; Thermo Fisher Scientific, Lenexa, KS), and phenotypic screening tests (cefoxotin) were used for the identification and isolation of MRSA. Vitek 2 AST-GP75 cards (bioMerieux, Durham, NC) were used to determine the susceptibility of *S. aureus* study isolates against ciprofloxacin, clindamycin, daptomycin, doxycycline, erythromycin, gentamicin, linezolid, mupirocin, rifampin, tetracycline, trimethoprim-sulfamethoxazole, and vancomycin. Double-disk diffusion tests were performed to identify inducible clindamycin resistance. Antimicrobial minimum inhibitory concentrations (MICs) were interpreted according to the Clinical and Laboratory Standards Institute document M100-S14 (2014).

*DNA sequencing and analyses:*

Bacterial DNA was extracted on the MagNA Pure 96 Instrument for automated DNA extraction using the Pathogen Universal 200 protocol (Roche Life Science). The quality of the extracted DNA was assessed with the Epoch Microplate Spectrophotometer (Biotek, Winooski, VT) and ran on an automated capillary electrophoresis system (Qiaxcel Advanced System; Qiagen, Valencia, CA). Genomic DNA was quantified using the high-sensitivity double stranded DNA

assay on the Qubit 2.0 fluorometer (Invitrogen, Life Technologies, Grand Island, NY). Sequencing libraries were prepared using the NexteraXT DNA sample preparation kit (Illumina Inc., San Diego, CA) following manufacturer's instructions. DNA libraries were sequenced on a MiSeq sequencing instrument (Illumina Inc., San Diego, CA) with 250-base paired-end reads.

Sequencing data were imported and analyzed using CLC Genomics Workbench 8.1 (Qiagen, Redwood City, CA). Paired-end reads were mapped to reference strain FPR3757 (accession NC_002952). FPR3757 was the first USA300, ST-8 strain to be completely sequenced; this strain was recovered from a skin abscess, and serves as a good reference strain for our comparative genome analysis.[102] Local realignment was performed to improve mapping in areas around insertions and deletions using an algorithm described by Homer et al.[205] The Fixed Ploidy Variant Detection tool was used to identify single nucleotide polymorphisms (SNPs) and Indels and Structural Variants tool was used to identify insertions and deletions.

Isolates with coverage levels $\geq$ 15x were included in the SNP analysis. An *in silico* SNP validation was performed to assess SNP frequency with varying coverage levels. Sequentially smaller sequence lists were randomly generated for a select isolate. Next, the aforementioned mapping and variant calling pipelines were run with the lower coverage subsets (Table 6.1). We identified that $\geq$ 15x coverage detected > 94% of quality SNPs.

**Table 6.1** *In silico* SNP validation

| Read Subsets | No of Reads | Average Coverage | Total SNPs | % SNPs Identified |
|---|---|---|---|---|
| 100% | 641775 | 25 | 68 | 100% |
| 90% | 577545 | 21 | 68 | 100% |
| 80% | 513323 | 19 | 67 | 99% |
| 70% | 449226 | 18 | 65 | 96% |
| 60% | 385327 | 14 | 64 | 94% |
| 50% | 320916 | 12 | 61 | 90% |

For comparative genome evaluation, SNPs were used as a measure of genetic pairwise distances between strains. A SNP matrix was generated using the Reference Sequence Alignment based Phylogeny builder (REALPHY: http://realphy.unibas.ch/fcgi/realphy) and inferred on PhyML. The alignment was uploaded onto MEGA7 and CLCGenomics Workbench 8.5.1 for phylogenetic analyses. Phylogeny was inferred using neighbor joining and maximum likelihood method based on the Jukes-Cantor model with 500 bootstrap replicates.

Multilocus sequence typing (MLST) was determined from the sequence data by extracting the sequence at the specific loci of the seven housekeeping genes using the CLCGenomics Workbench 8.5.1 and the *S. aureus* MLST scheme (www.pubmlst.org; downloaded November 2015).

To assemble the virulomes, the presence of known virulence genes were detected using local blast against the *S. aureus* VirulenceFinder v1.5 database. The database contained 194 virulence genes classified as exoenzymes, host immune evasion, and toxins.

*Genome-wide association analysis:*

GWAS aims to discover variants occurring over the genome that could, in isolation or in combination, lead to a particular trait or an unfortunate phenotype such as a disease. The basic premise of GWAS is a case-control study to statistically analyze the genetic differences between two populations: unaffected (controls) vs. affected individuals (cases). We conducted a case-control association analysis on a set of 30 colonizing isolates and 31 SSTI isolates to identify SNPs that were significantly associated with clinical syndrome. Significance levels were corrected for multiple tests using the false discovery rate (FDR) procedure. An FDR threshold of 10% was used to indicate significance. GWAS is sensitive to bias due to population structure, leading to false positives. To account for population stratification, we performed a hierarchical clustering approach to estimate the population structures.[229] Next, we performed a second series of tests on specific variants conditioned on the results of the population structure using the Cochran-Mantel-Haenszel test.[232] We estimated the impact of the population structure in the reduction of false positive associations using the genomic inflation factor. The inflation factor is a parameter defined as the ratio of observed distribution of the chi statistic to the expected median.[233] A high inflation factor generally indicates a high rate of false positives biased by influence of underlying population structure.

*Statistical analyses:*

Descriptive statistics were used to describe patient characteristics and the genetic profile of the bacterial isolates. The Chi-square test or Fisher's Exact test was used for dichotomous or categorical variables. The Student's t test or Wilcoxon rank sum test was used for continuous variables. An alpha level of 0.5 was used to detect statistical significance. Statistical analyses were performed using SPSS 23.0® and CLC Genomics Software 7.0 (Qiagen, Valencia, CA).

*Predictive modeling:*

The standard approach to GWAS is based on univariate analyses and does not directly account for correlations among explanatory variables or provide predictive risk models.[31] These limitations can be addressed by advanced multivariate statistics or machine learning techniques. To build a predictive model for SSTI, we used a Random Forest method using SPSS 18.0® Modeler (IBM Corp, Armonk NY).[228-230,234] Random Forest is an ensemble machine learning method, which constructs many decision trees used to classify a new instance by the majority vote.[234,235] Each decision tree node uses a subset of variables randomly selected from the whole original set of variables. Each tree uses a different bootstrap sample data. For the model, we included SNPs, indels, and clinical features detected in the univariate models. Importance scores for each of the variables for distinguishing the phenotypes were generated. The model was run using the parameters (bin=10 and ntree=100). In the Random Forest

approach, the initial cohort is divided into “in bag” and “out-of-bag” samples. The in-bag sample is created using random sampling with replacement from the initial cohort, creating a sample equivalent in size as the initial cohort. The out-of-bag sample is composed of un-sampled data from the initial cohort, and included one-third of the initial cohort. The out-of-bag cohort served as an internal validation cohort for the model derived using the “in-bag” sample. The proportions of accurate predictions of phenotypes were reported.

## *Results*

### *Clinical and epidemiological features of patients with S. aureus SSTI and colonization*

The clinical characteristics of the individuals with *S. aureus* SSTIs and nasal colonization are shown in Table 6.2. Overall, the cohort had an average age of 41 years (SD $\pm$ 13), approximately half were male, and most were Hispanic/Latino (78%). A significantly higher proportion of nasal carriers reported being healthcare providers ($p<0.01$) while a significantly higher proportion of SSTI patients reported having a history of a prior skin infection ($p=0.05$). All other characteristics were similar between the two groups.

**Table 6.2** Characteristics of patients with community-associated *S. aureus* SSTI compared to *S. aureus* nasal colonization

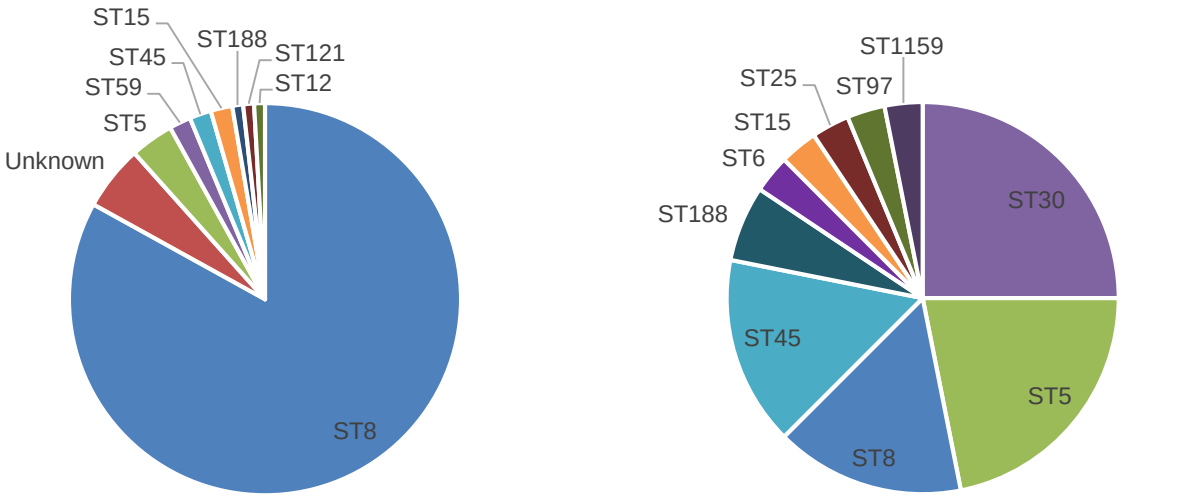
| Characteristic | SSTI n = 112 | Colonization n = 32 | OR (95% CI) | *P* |
|---|---|---|---|---|
| Mean age, yrs. (±SD) | 41 (±13) | 41 (±13) | | 0.59 |
| Male | 56 (50%) | 17 (53%) | 0.90 (0.49-1.67) | 0.76 |
| Race/Ethnicity | | | | |
| Black | 6 (5%) | 2 (6%) | 0.88 (0.26-3.05) | 1.00 |
| Hispanic | 85 (76%) | 27 (84%) | 0.65 (0.27-1.55) | 0.31 |
| Diabetes | 31 (28%) | 10 (31%) | 0.88 (0.46-1.68) | 0.69 |
| Obese (BMI ≥ 30) | 61 (55%) | 18 (56%) | 0.95 (0.51-1.75) | 0.86 |
| Weight ≥ 110 kg | 26 (23%) | 5 (16%) | 1.48 (0.62-3.55) | 0.36 |
| Peripheral vascular disease | 0 | 0 | n/a | |
| Chronic skin disorder | 1 (0.9%) | 0 | 1.02 (0.98-1.06) | 1.00 |
| HIV | 0 | 0 | n/a | |
| Cancer | 0 | 0 | n/a | |
| Chemotherapy | 0 | 0 | n/a | |
| Healthcare provider | 1 (0.9%) | 4 (12.5%) | 0.25 (0.15-0.44) | 0.01* |
| History of skin infection | 28 (25%) | 3 (9%) | 2.65 (0.87-8.13) | 0.05* |
| Prior Antibiotics within 90days | 14 (13%) | 5 (16%) | 0.82 (0.36-1.87) | 0.77 |

SSTI= skin and soft tissue infection; OR=odds ratio; CI=confidence interval; yrs.=years; SD=standard deviation; BMI=body mass index; HIV=human immunodeficiency virus. (*) indicates statistical significance.

*Heterogeneity of S. aureus SSTI and colonizing strains*

We sequenced 144 *S. aureus* isolates (112 from SSTIs and 32 from nasal colonization) recovered from 144 patients presenting to a STARNet primary care clinic. We found significant differences in the distribution of strain-types between strains isolated from nasal carriers and those isolated from SSTIs (Figure 6.1). A significantly higher proportion of the SSTI strains were ST8 compared to carrier strains (83% vs 16%; $p<0.01$). Comparatively, a significantly higher proportion of carrier strains were ST30 (8% vs. 0%, $p<0.01$), ST45 (16% vs. 2%; $p<0.01$), and ST5 (22% vs 4%; $p<0.01$) compared to SSTI strains.

**Figure 6.1** Multi-locus sequence typing of *S. aureus* isolates from SSTIs and nasal colonization




*S. aureus* SSTI isolates (n=112)

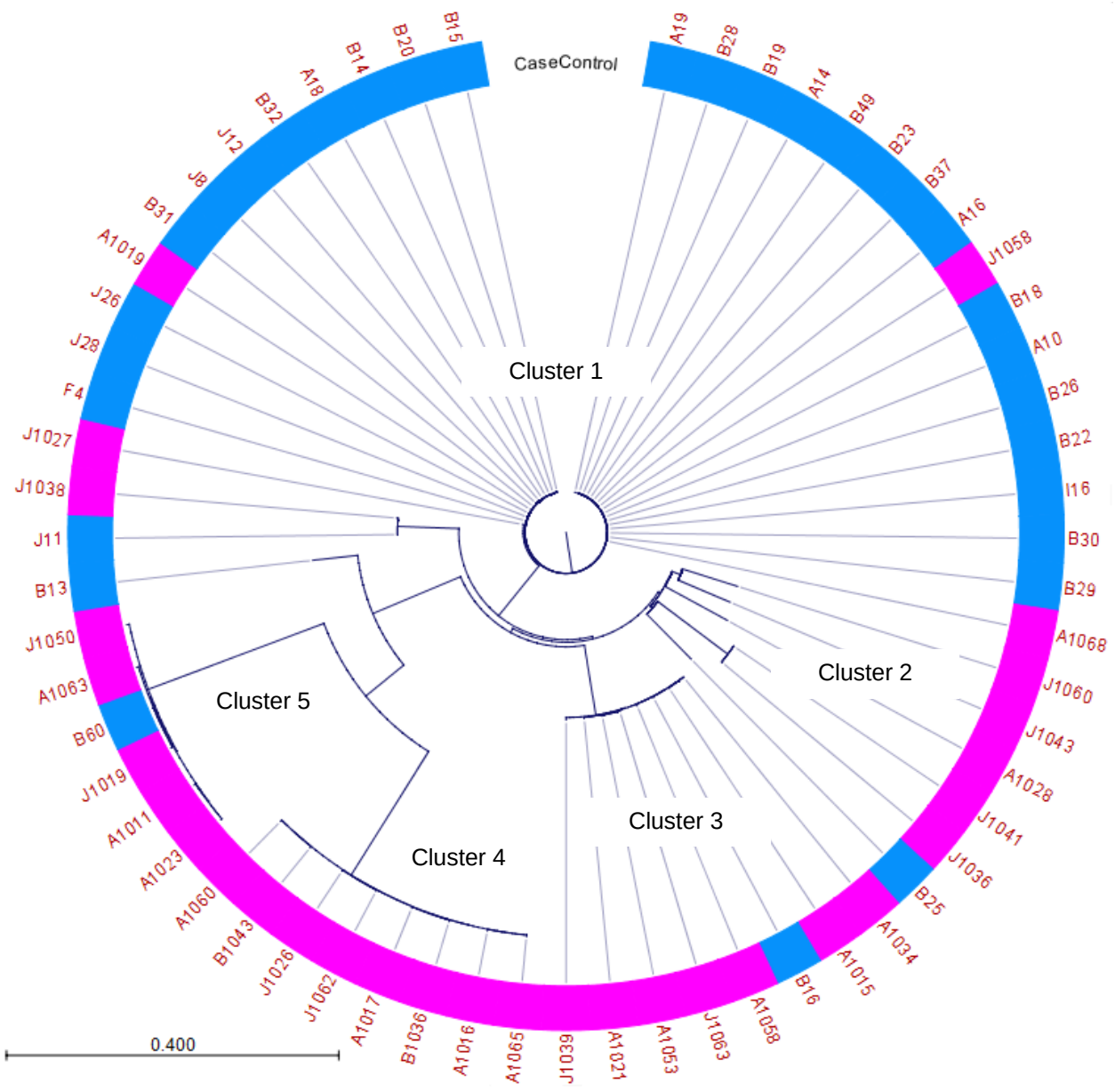
*S. aureus* colonization isolates (n=32)

SSTI=skin and soft tissue infection

*SNP-based phylogenetic analysis and genomic diversity of S. aureus SSTI and colonizing strains*

A genome wide phylogenetic analysis was conducted based on the SNPs in the core genomes of 61 *S. aureus* isolates. Overall, the strains differed by an average of 16,090 SNPs from the reference FPR3757. There were 5 main clusters. The first cluster comprised of mostly ST-8 SSTI isolates (Figure 6.2). Clusters 2 and 4 comprised of all colonizing isolates, and clusters 3 and 5 contained both SSTI and colonizing isolates. The majority of the SSTI isolates (in clusters 1, 3, and 5) clustered closely with the reference FPR3757 and had an average of 4,469 SNPs when compared to the reference. Comparatively, the nasal colonization strains were more divergent from the reference, with an average of 28,100 SNPs. To assess how the clinical phenotypes are distributed across the genetic variability that exists within this collection of isolates, we mapped the phenotype onto a phylogenetic tree based on the genome sequences of these isolates, showing the distribution of SSTI and colonizing phenotypes across the genotypes as well as some clustering.

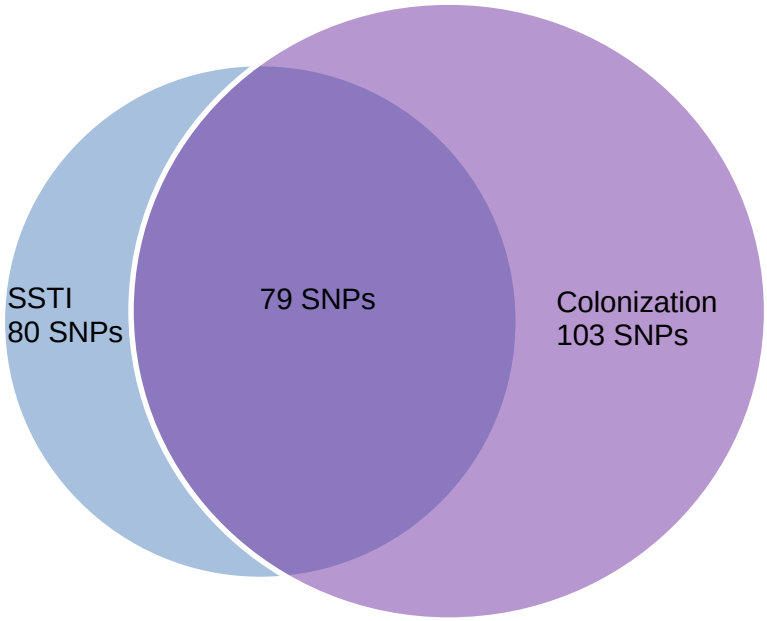
**Figure 6.2** SNP-based phylogenetic analyses



*SNP analysis of ST8 strains involved in colonization compared to SSTIs*

We conducted a SNP analysis comparing ST8 nasal colonization strains and SSTI strains. Within group SNPs were identified if the variants were present in at least 70% of the samples with the phenotype. When compared to the reference genome, there were 79 overlapping SNPs among ST8 *S. aureus* SSTI and colonization isolates (Figure 6.3). There were 25 unique SNPs, of which 24 were among the colonization strains. Of these 24 SNPs, 9 were nonsynonymous, 4 were synonymous, and 11 occurred in intergenic regions. Notably, 3 of the nonsynonymous changes occurred in loci associated with antimicrobial activity including *gyrA*, *parC*, and an antibiotic transport protein (SAUSA300_rs13825).

**Figure 6.3** Venn diagram comparing SNPs of ST8 *S. aureus* isolates involved in colonization versus SSTI




SSTI=skin and soft tissue infection; SNPs=single nucleotide polymorphisms
Blue indicates SSTI isolates and purple indicates colonization isolates. Within group SNPs were identified if the variants were present in at least 70% of the samples. There were a total 79 overlapping SNPs and 25 unique SNPs between the *S. aureus* isolate groups when compared to the reference genome, FPR3757.

*Virulome*

All *S. aureus* isolates contained an average of 43 virulence genes tested. There were no differences in the number of virulence genes harbored among *S. aureus* SSTI isolates compared to colonization isolates (43 vs. 42; $p$=0.69). Overall frequencies of virulence genes are shown in Table 6.3. Common virulence genes detected across the isolates included *aur* (100%), *hlb* (98%), *hlgB* (98%), *scn* (94%), *hlgA* (92%), *hlgC* (90%), *sak* (90%), *lukE* (85%), *lukD* (83%), and *splA* (81%). Less than 10% of the isolates tested carried *sed* (7%), *sej* (7%), *ser* (7.0%), *tst* (6%), *seb* (4%), *sel* (2%), *sec3* (1%), and *edin* (1%). While the invasiveness of *S. aureus* was thought to largely depend on the carriage of a battery of virulence factors, in our study we found that number of virulence genes was not associated with pathogenicity.

The distribution of some virulence genes, especially enterotoxin genes (*sea, sep*, *seb, sed*, *seg, sei, sej, sek, sem, sen, seo, seq, ser, seu)*, were correlated with different *S. aureus* lineages (Table 6.3).There were differences in the carriage of certain adhesion factors including *splA*, *splB*, *splE*. The carriage of *pvl* genes were identified only among ST8 strains and in one ST121 strain. There were no differences in the carriage of gamma hemolysins across the different lineages. The *tst* gene was only found among nasal colonizing strains belonging to ST30. These differences in the carriage rates of virulence genes among different ST isolates suggest that different *S. aureus* lineages associated with SSTIs have specific patterns of virulence genes.

**Table 6.3** Virulence genes by multilocus sequence strain types

| Virulence Factor | Overall | ST8 (n=98) | ST5 (n=11) | ST30 (n=8) | ST45 (n=7) | Unkwn (n=6) | ST15 (n=3) | ST59 (n=2) | ST188 (n=3) | ST1159 (n=1) | ST121 (n=1) | ST97 (n=1) | ST6 (n=1) | ST12 (n=1) | ST25 (n=1) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Adhesions** (n, %) | | | | | | | | | | | | | | | |
| *aur* (n, %) | 144 (100) | 98 (100) | 11 (100) | 8 (100) | 7 (100) | 6 (100) | 3 (100) | 2 (100) | 3 (100) | 1 (100) | 1 (100) | 1 (100) | 1 (100) | 1 (100) | 1 (100) |
| *fnbA* | 142 (98.6) | 96 (98) | 11 (100) | 8 (100) | 7 (100) | 6 (100) | 3 (100) | 2 (100) | 3 (100) | 1 (100) | 1 (100) | 1 (100) | 1 (100) | 1 (100) | 1 (100) |
| *fnbB* | 134 (93) | 91 (98) | 11 (100) | 8 (100) | 4 (57) | 6 (100) | 3 (100) | 2 (100) | 3 (100) | 1 (100) | 1 (100) | 1 (100) | 1 (100) | 1 (100) | 1 (100) |
| *clfA* | 144 (100) | 98 (100) | 11 (100) | 8 (100) | 7 (100) | 6 (100) | 3 (100) | 2 (100) | 3 (100) | 1 (100) | 1 (100) | 1 (100) | 1 (100) | 1 (100) | 1 (100) |
| *clfB* | 144 (100) | 98 (100) | 11 (100) | 8 (100) | 7 (100) | 6 (100) | 3 (100) | 2 (100) | 3 (100) | 1 (100) | 1 (100) | 1 (100) | 1 (100) | 1 (100) | 1 (100) |
| *icaA* | 144 (100) | 98 (100) | 11 (100) | 8 (100) | 7 (100) | 6 (100) | 3 (100) | 2 (100) | 3 (100) | 1 (100) | 1 (100) | 1 (100) | 1 (100) | 1 (100) | 1 (100) |
| *icaD* | 144 (100) | 98 (100) | 11 (100) | 8 (100) | 7 (100) | 6 (100) | 3 (100) | 2 (100) | 3 (100) | 1 (100) | 1 (100) | 1 (100) | 1 (100) | 1 (100) | 1 (100) |
| *splA* | 117 (81.3) | 89 (90.8) | 11 (100) | 0 | 0 | 6 (100) | 3 (100) | 0 | 3 (100) | 1 (100) | 0 | 1 (100) | 1 (100) | 1 (100) | 1 (100) |
| *splB* | 108 (75.0) | 80 (81.6) | 11 (100) | 0 | 0 | 5 (83.3) | 3 (100) | 0 | 3 (100) | 1 (100) | 1 (100) | 1 (100) | 1 (100) | 1 (100) | 1 (100) |
| *splE* | 112 (77.8) | 88 (89.8) | 0 | 8 (100) | 0 | 5 (83.3) | 3 (100) | 0 | 3 (100) | 1 (100) | 0 | 1 (100) | 1 (100) | 1 (100) | 1 (100) |
| *splF* | 121 (84.0) | 89 (90.8) | 11 (100) | 8 (100) | 0 | 6 (100) | 3 (100) | 0 | 3 (100) | 1 (100) | 0 | 1 (100) | 1 (100) | 1 (100) | 1 (100) |
| **Host immune evasins** (n, %) | | | | | | | | | | | | | | | |
| *ACME* | 58 (40) | 54 (55.1) | 0 | 0 | 0 | 4 (66.7) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *edinA* | 1 (0.7) | 0 | 1 (9.7) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *edinB* | 1 (0.7) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 (100) |
| *sak* | 125 (86.8) | 87 (88.8) | 11 (100) | 5 (62.5) | 7 (100) | 6 (100) | 0 | 0 | 3 (100) | 1 (100) | 1 (100) | 1 (100) | 1 (100) | 1 (100) | 1 (100) |
| *scn* | 135 (93.8) | 93 (94.9) | 11 (100) | 4 (50.0) | 7 (100) | 6 (100) | 3 (100) | 2 (100) | 3 (100) | 1 (100) | 1 (100) | 1 (100) | 1 (100) | 1 (100) | 1 (100) |
| **Toxins** (n, %) | | | | | | | | | | | | | | | |
| *Enterotoxin* | 14 (9.7) | 2 (2.0) | 8 (72.7) | 0 | 3 (42.9) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 (100) |
| *hla* | 142 (98.6) | 96 (97.9) | 11 (100) | 8 (100) | 7 (100) | 6 (100) | 3 (100) | 2 (100) | 3 (100) | 1 (100) | 1 (100) | 1 (100) | 1 (100) | 1 (100) | 1 (100) |
| *hlb* | 141 (97.9) | 95 (96.9) | 11 (100) | 8 (100) | 7 (100) | 6 (100) | 3 (100) | 2 (100) | 3 (100) | 1 (100) | 1 (100) | 1 (100) | 1 (100) | 1 (100) | 1 (100) |
| *hlgA* | 133 (92.4) | 88 (89.8) | 11 (100) | 8 (100) | 7 (100) | 6 (100) | 2 (66.7) | 2 (100) | 3 (100) | 1 (100) | 1 (100) | 1 (100) | 1 (100) | 1 (100) | 1 (100) |
| *hlgB* | 141 (97.9) | 96 (98.0) | 11 (100) | 8 (100) | 7 (100) | 5 (83.3) | 3 (100) | 2 (100) | 3 (100) | 1 (100) | 1 (100) | 1 (100) | 1 (100) | 1 (100) | 1 (100) |
| *hlgC* | 130 (90.3) | 88 (89.8) | 11 (100) | 8 (100) | 7 (100) | 3 (50) | 2 (66.7) | 2 (100) | 3 (100) | 1 (100) | 1 (100) | 1 (100) | 1 (100) | 1 (100) | 1 (100) |
| *lukD* | 120 (83.3) | 93 (94.9) | 11 (100) | 0 | 0 | 5 (83.3) | 3 (100) | 0 | 3 (100) | 1 (100) | 1 (100) | 0 | 1 (100) | 1 (100) | 1 (100) |
| *lukE* | 123 (85.4) | 96 (98.0) | 11 (100) | 0 | 0 | 5 (83.3) | 3 (100) | 0 | 3 (100) | 1 (100) | 1 (100) | 0 | 1 (100) | 1 (100) | 1 (100) |
| *lukF* | 92 (63.9) | 87 (88.8) | 0 | 0 | 0 | 4 (66.7) | 0 | 0 | 0 | 0 | 1 (100) | 0 | 0 | 0 | 0 |
| *lukS* | 78 (54.2) | 74 (75.5) | 0 | 0 | 0 | 3 (50) | 0 | 0 | 0 | 0 | 1 (100) | 0 | 0 | 0 | 0 |
| *sea/sep* | 14 (9.7) | 2 (2.0) | 2 (18.8) | 4 (50.0) | 0 | 1 (16.7) | 0 | 0 | 2 (66.7) | 1 (100) | 0 | 0 | 1 (100) | 1 (100) | 0 |
| *seb* | 6 (4.2) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 (66.7) | 0 | 0 | 0 | 0 | 1 (100) | 1 (100) |

**Table 6.3** Virulence genes by multilocus sequence strain types (continued)

| Virulence Factor | Overall | ST8 (n=98) | ST5 (n=11) | ST30 (n=8) | ST45 (n=7) | Unkn (n=6) | ST15 (n=3) | ST59 (n=2) | ST188 (n=3) | ST1159 (n=1) | ST121 (n=1) | ST97 (n=1) | ST6 (n=1) | ST12 (n=1) | ST25 (n=1) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Toxins cont'd** | | | | | | | | | | | | | | | |
| *sec3* | 2 (1.4) | 1 (1.0) | 0 | 0 | 1 (14.3) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *sed* | 10 (6.9) | 2 (2.0) | 8 (72.7) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *seg* | 29 (20.1) | 1 (1.0) | 11 (100) | 8 (100) | 7 (100) | 0 | 0 | 0 | 0 | 0 | 1 (100) | 0 | 0 | 0 | 1 (100) |
| *sei* | 27 (18.8) | 2 (2.0) | 8 (72.7) | 8 (100) | 7 (100) | 0 | 0 | 0 | 0 | 0 | 1 (100) | 0 | 0 | 0 | 1 (100) |
| *sej* | 10 (6.9) | 2 (2.0) | 8 (72.7) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *sek* | 86 (59.7) | 79 (80.6) | 1 (9.1) | 0 | 0 | 4 (66.7) | 0 | 2 (100) | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *sel* | 3 (2.1) | 2 (2.0) | 0 | 0 | 1 (14.3) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *sem* | 28 (19.4) | 2 (2.0) | 10 (90.9) | 8 (100) | 6 (85.7) | 0 | 0 | 0 | 0 | 0 | 1 (100) | 0 | 0 | 0 | 1 (100) |
| *sen* | 28 (19.4) | 2 (2.0) | 11 (100) | 8 (100) | 5 (71.4) | 0 | 0 | 0 | 0 | 0 | 1 (100) | 0 | 0 | 0 | 1 (100) |
| *seo* | 28 (19.4) | 1 (1.0) | 11 (100) | 8 (100) | 7 (100) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 (100) |
| *seq* | 90(62.5) | 81 (82.7) | 2 (18.8) | 0 | 0 | 5 (83.3) | 0 | 2 (100) | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *ser* | 10 (6.9) | 2 (2.0) | 8 (72.7) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *seu* | 15 (10.4) | 0 | 3 (27.3) | 8 (100) | 3 (42.9) | 0 | 0 | 0 | 0 | 0 | 1 (100) | 0 | 0 | 0 | 0 |
| *tst* | 9 (6.3) | 0 | 2 (18.8) | 7 (87.5) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

We compared the differences in carriage of virulence genes of SSTI and colonization strains among ST8 lineage (Table 6.4). The *hlb* gene was present in 99% of the SSTI strains compared to 60% of the colonizing strains ($p<0.01$). Prior studies have described the well-established role of *S. aureus hlb* in skin colonization by damaging keratinocytes, in addition to its well-known hemolytic activity for erythrocytes.[35] There were no other differences in virulence genes identified.

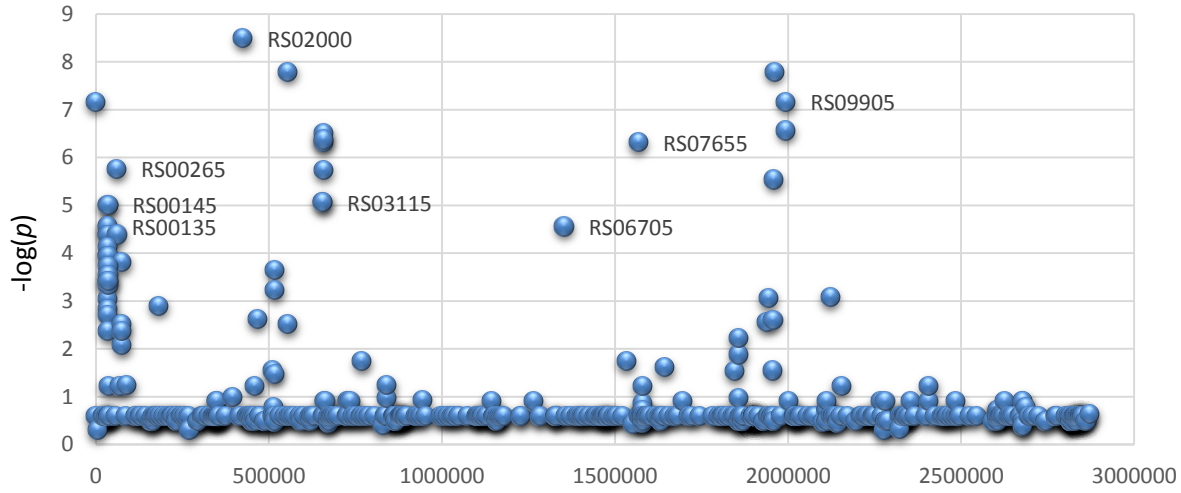
**Table 6.4** Virulence genes among ST8 *S. aureus* SSTI and colonization isolates

| Virulence Factor | SSTI n=93 | Colonization n=5 | *P* |
|---|---|---|---|
| **Adhesions** | | | |
| *aur* | 93 (100) | 5 (100) | n/a |
| *fnbA* | 91 (98) | 5 (100) | n/a |
| *fnbB* | 91 (98) | 5 (100) | n/a |
| *clfA* | 93 (100) | 5 (100) | n/a |
| *clfB* | 93 (100) | 5 (100) | n/a |
| *icaA* | 93 (100) | 5 (100) | n/a |
| *icaD* | 93 (100) | 5 (100) | n/a |
| *splA* | 84 (90.3) | 5 (100) | 1.00 |
| *splB* | 75 (80.6) | 5 (100) | 0.58 |
| *splE* | 82 (88.1) | 5 (100) | 0.69 |
| *splF* | 85 (95.5) | 4 (80) | 0.46 |
| **Host immune evasins** | | | |
| *ACME* | 53 (57) | 1 (20) | 0.17 |
| *edinA* | 0 | 0 | n/a |
| *edinB* | 0 | 0 | n/a |
| *sak* | 82 (88.2) | 5 (100) | 1.00 |
| *scn* | 87 (93.1) | 5 (100) | 1.00 |
| **Toxins** | | | |
| *Enterotoxin* | 2 (2.2) | 0 (0) | 1.00 |
| *hla* | 92 (99) | 4 (80.0) | 0.10 |
| *hlb* | 92 (99) | 3 (60.0) | <0.01* |
| *hlgA* | 83 (89.2) | 5 (100.0) | 1.00 |
| *hlgB* | 92 (98.9) | 4 (80.0) | 0.10 |
| *hlgC* | 84 (90.3) | 4 (80.0) | 0.42 |
| *lukD* | 88 (94.6) | 5 (100) | 1.00 |
| *lukE* | 89 (95.7) | 5 (100) | 0.84 |
| *lukF* | 84 (90.3) | 3 (60.0) | 0.09 |
| *lukS* | 72 (77.4) | 2 (40.0) | 0.09 |
| *sea/sep* | 1 (1.1) | 1 (20.0) | 0.10 |
| *seb* | 0 | 0 | n/a |
| *sec3* | 1 (1.1) | 0 | 1.00 |
| *sed* | 1 (1.1) | 1 (20.0) | 0.10 |
| *seg* | 1 (1.1) | 0 | 1.00 |
| *sei* | 2 (2.2) | 0 | 1.00 |
| *sej* | 1 (1.1) | 1 (20.0) | 0.10 |
| *sek* | 75 (80.6) | 4 (80.0) | 1.00 |
| *sel* | 1 (1.1) | 1 (20.0) | 0.10 |
| *sem* | 2 (2.2) | 0 | 1.00 |
| *sen* | 2 (2.2) | 0 | 1.00 |
| *seo* | 1 (1.1) | 0 | 1.00 |
| *seq* | 77 (82.8) | 4 (80.0) | 1.00 |
| *ser* | 1 (1.1) | 1 (20.0) | 0.10 |
| *seu* | 0 | 0 | n/a |
| *tst* | 0 | 0 | n/a |

*Genome-wide comparison of S. aureus SSTI and colonization isolates*

We compared the genomes of 31 SSTIs and 30 nasal colonization *S. aureus* isolates to identify the genetic polymorphisms associated with the clinical phenotype. Out of a total of 529 SNPs, we identified 18 SNPs that were significantly associated with SSTIs after adjustment using FDR (Figure 6.4). Further, 3 indels were found to be significant. These SNPs and indels were distributed across the genome in 8 candidate loci among mobile genetic elements (SCC*mec* and ACME), genes involved in metabolism and regulation, RNA modification enzymes, and in genes encoding for hypothetical proteins. Six were nonsynonymous, 4 were synonymous, and 11 were in intergenic regions.

**Figure 6.4** Manhattan plot of *S. aureus* SSTI genome-wide study




SSTI=skin and soft tissue infection. Manhattan plot summarizes the association of whole genome variants with SSTIs compared to colonization *S. aureus* isolates as well as particular coding regions which showed strong associations. The y-axis represents the statistical significance of association for each variant in order on the genome (x-axis).Genes with significant associations are annotated.

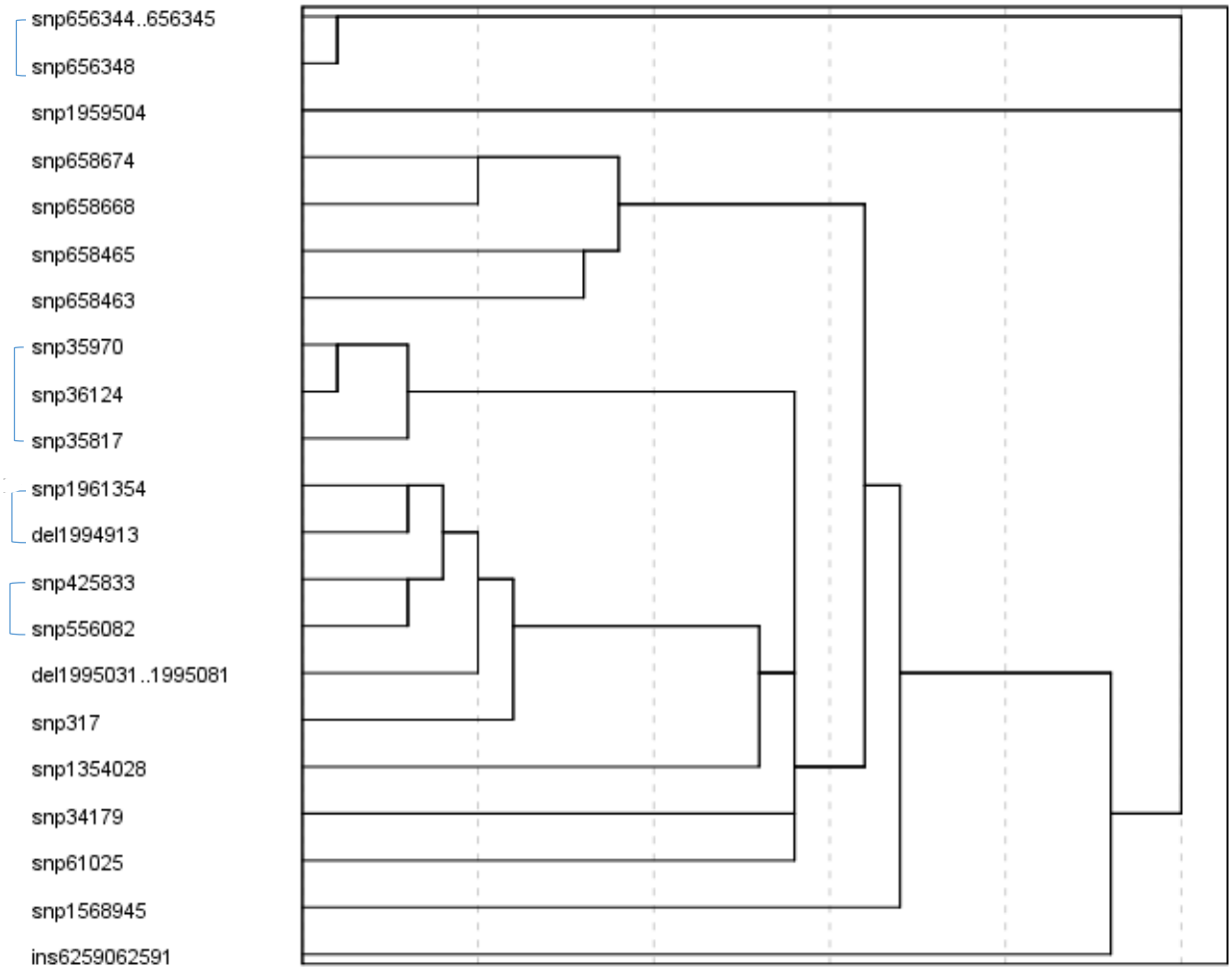
The description of these SNPs and functions of the loci is located in Table 6.5. Hierarchical clustering analysis based on the SNP/indel proximity identified potential linkage disequilibrium of variants; however, many appeared to be independent (Figure 6.5).

The intrinsic clonal population structure can result in high false positive rates in genome wide association studies.[30] To estimate the population structures, we used a hierarchical clustering algorithm that showed 6 main clusters (Figure 6.6). Based on this clustering information, the Cochran-Mantel Haenszel association statistic was used to test for associations between SSTI and specific variants, conditioned on the population cluster. Adjusting for the population structure decreased the genomic inflation factor from 54.8 (median chi-squared statistic=24.9) to 2.5 (median chi-squared statistic=1.15). This decrease indicates a reduction in the probability of false positive rates due to population substructures. Out of the 21 polymorphisms previously identified, only 3 (snp425833, snp1568945, and ins62590^62591) remained significant after this procedure. Unfortunately, the limited sample size prevented us from using a more detailed clustering approach. One of these candidate loci (snp425833) was located in a gene encoding for phosphoglycerate mutase, an enzyme responsible for glycolysis (RS02000), which results in Glu106Asp change to the amino acid sequence. The second SNP was located in a gene encoding a phage portal protein (RS07655). The third candidate was located in an intergenic region between a transposase and the *speG* gene within the ACME unit.

**Table 6.5** Details of significantly associated variants

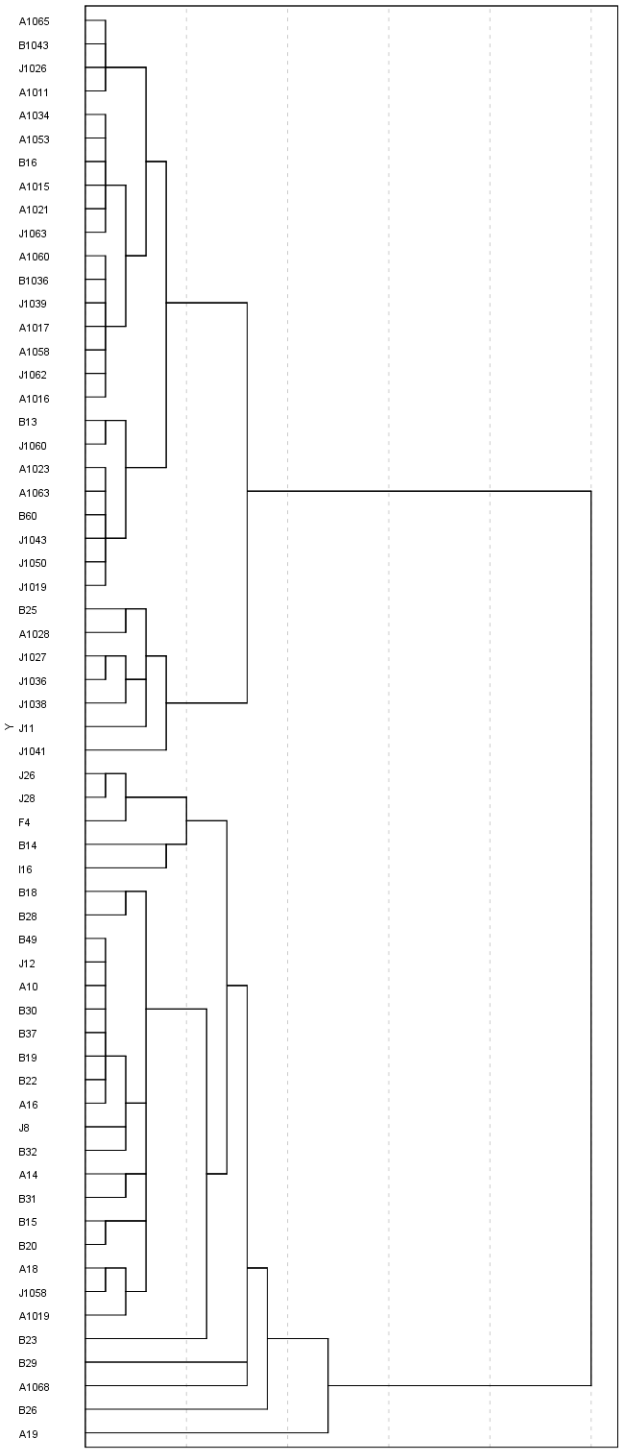
| Position in reference FPR3757 | Reference base | Allele | Type | Effect | CDS in FPR3757 | CDS Function |
|---|---|---|---|---|---|---|
| 425833 | A | T | SNV | Nonsynonymous | RS02000 | Phosphoglycerate mutase - metabolism |
| 556082 | T | C | SNV | Noncoding | Intergenic | Flanked by a lysyl-tRNA synthetase (*lysS*) and transcriptional regulator protein, (*gntR*) |
| 1961354 | C | T | SNV | Noncoding | Intergenic | Flanked by alpha/beta hydroxylase and a hypothetical protein |
| 317 | A | G | SNV | Noncoding | Intergenic | Flanked by chromosomal replication initiator protein *dnaA* |
| 1994913 | A | - | Deletion | Nonsynonymous | RS09905 | IS1181, transposase |
| 658674 | T | G | SNV | Noncoding | Intergenic | Flanked by two hypothetical proteins |
| 1995031..1995081 | TTTTAAAAATAG TTCTTTAAATTAT ATACCCACCAC ATTTGGTGGAG AACC | - | Deletion | Noncoding | Intergenic | Flanked by a transposase and a transcriptional regulator, peroxide-responsive repressor *perR* |
| 658465 | T | A | SNV | Noncoding | Intergenic | Flanked by two hypothetical proteins |
| 658668..658672 | TTTTT | GAGCC | MNV | Noncoding | Intergenic | Flanked by two hypothetical proteins |
| 1568945 | C | T | SNV | Synonymous | RS07655 | Phage portal protein |
| 61025 | G | A | SNV | Nonsynonymous | RS00265 | Hypothetical protein |
| 658463 | A | T | SNV | Noncoding | Intergenic | Flanked by two hypothetical proteins |
| 1959504 | T | A | SNV | Noncoding | Intergenic | Flanked by alpha/beta hydroxylase and a hypothetical protein |
| 35970 | T | C | SNV | Synonymous | RS00145 | Putative transposase; K07498 |
| 36124 | G | A | SNV | Nonsynonymous | RS00145 | Putative transposase; K07498 |
| 656344..656345 | AA | TT | MNV | Nonsynonymous | RS03115 | Hypothetical protein |
| 656348 | G | T | SNV | Nonsynonymous | RS03115 | Hypothetical protein |
| 34179 | T | A | SNV | Synonymous | RS00135 | Ribosomal RNA large subunit methyltransferase H; 23S rRNA modification factors |
| 35817 | A | T | SNV | Noncoding | Intergenic | Flanked between a transposase and a hypothetical protein |
| 1354028 | C | T | SNV | Synonymous | RS06705 | Secretion protein |
| 62590^62591 | - | A | Insertion | Noncoding | Intergenic | Flanked between a transposase and spermidine N1-acetyltransferase (*speG*) |

**Figure 6.5 Evaluation of potential linkage disequilibrium**




Tree showing all SNPs and indels, with those in linkage disequilibrium clustered together with blue bracket.

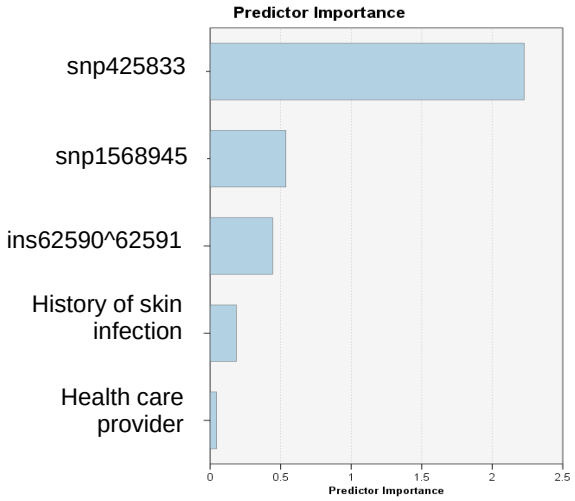
**Figure 6.6** Hierarchical clustering of isolates



*Predictive modeling*

To test whether these candidate signatures can be predictive of *S. aureus* SSTIs, we built a predictive model using the set of 5 variables [3 SNPs/ins and 2 clinical predictors (health care provider status and history of prior skin infection)]. The model showed an accuracy of >88% (misclassification rate of <12%). The phenotype prediction rate is shown in Table 6.6. The significance of each variable in this model is displayed on Figure 6.7. The top influential variable was snp425833.

**Figure 6.7** Random forest predictor importance




Variables ordered by descending degree of importance in the predictive model.

**Table 6.6** Random Forest prediction of SSTIs

| Observed | Predicted | | |
|---|---|---|---|
| | Colonization | SSTI | % Correct |
| Colonization | 26 | 5 | 84% |
| SSTI | 3 | 26 | 90% |

***Discussion***

There has been an increase in the incidence of community-associated *S. aureus* infections worldwide.[8,59,236] SSTIs are the most common manifestation of CA-MRSA infections; however, the molecular changes and contribution of select genetic features of *S. aureus* that facilitate its propensity to cause SSTIs has yet to be determined. The application of WGS captures the full extent of bacterial genomic variations and allows for genome-wide comparisons can potentially be applied to predict the pathogenicity of staphylococcal infections.[237,238] In this study, we have described the bacterial genomic heterogeneity among *S. aureus* from the South Texas community setting associated with colonization and skin infections.

Prior studies have described the contribution of a particular *S. aureus* clonal type to be associated with disease manifestations.[2,39,77,175,176,209,211,239] Consistent with prior literature, we found that ST8 genotype is associated with SSTIs and that the nasal carriage rate of ST8 remains relatively low.[91,160,163,164,225] In addition, we found that the CA-MRSA colonization rates (3%) are far below CA-MSSA (20%). This may be the nature of a combination of factors including colonization site(s) not evaluated (e.g. skin, nasopharynx, gastrointestinal), different risk of person-to-person spread from infected patients or from contaminated fomites, and unique pathogenicity mechanisms that may bypass the hypothesis of sequential colonization before infection. Although our study demonstrated a significant association between genotype and SSTIs, it is important to note that almost all of

the common clonal types were found in both SSTIs and nasal colonization isolates. Hence, most *S. aureus* strains have the capacity to cause SSTIs.

There was diversity in the distribution of virulence genes among the strain types. Interestingly, the nasal colonization strains carried a similar array of virulence genes as those strains isolated from SSTIs. This may imply that rather than large scale genomic changes at the gene level, higher resolution changes at the nucleotide level may be driving the pathogenic potential of *S. aureus* strains.

With the genome sequences of both SSTI and nasal colonization strains, we conducted a bacterial GWAS to determine potential genetic markers down to the single nucleotide level associated with the pathogenicity of community-associated *S. aureus* strains. We identified 22 significant SNPs, of which 3 remained significant when population structure was considered in the model. Among these 3 variations, 1 non-synonymous SNP (snp425833) was found to be the most influential variable in the predictive model for SSTIs. The candidate SNP was located in a gene encoding for phosphoglycerate mutase. Phosphoglycerate mutase is an important surface enzyme in glycolysis and gluconeogenesis.[240] Prior studies have described the multiple roles of glycolytic and metabolic enzymes and their role in virulence characteristics.[240,241] Phosphoglycerate mutase has been previously identified as a streptococcal cell surface plasminogen binding protein.[242] In addition, *in vitro* studies have identified phosphoglycerate mutase as an antigen that is upregulated in staphylococcal biofilm production; Beenken et al. found phosphoglycerate mutase was expressed at higher levels in biofilms than in

planktonic *S. aureus*.[240,243] This reveals a potential novel mechanism of pathogenicity and transmission as biofilm formation by *S*. *aureus* is believed to contribute to their ability to colonize both biotic and abiotic surfaces. The functional role of phosphoglycerate mutase in the pathogenesis of skin infections requires further exploration. The second most influential site was an insertion in an intergenic region between a transposase and the *speG* gene. The *speG* gene in the arginine catabolic mobile element (ACME) unit encodes a spermidine acetyltransferase that allows *S. aureus* to evade polyamines, products involved in wound healing and inflammation on human tissues.[144-146] The third most influential loci was within a gene encoding for a phage portal protein. Further work is needed to characterize these loci and whether it may lead to amplification or attenuation of *S. aureus* pathogenesis cascade.

There were several limitations to this study. First, an important limitation is the small sample size which may have limited the identification of significant SNPs or SNP-SNP interactions with lower effect sizes. Second, SNPs could represent variable and transient events within a bacterial species, therefore spontaneous mutations cannot be entirely ruled out. However, other studies have suggested that genomic variation between closely related bacterial strains have undergone selection and are not spontaneous mutations.[244] Third, we identified a relatively high genome inflation factor suggesting that the underlying clonal population structure remains an issue that may lead to false positives. We attempted to address this confounding by conducting a series association tests conditioned for

the population structure; however, residual confounding may remain. While a within-host evolutionary study may have been more ideal to identify causality, the nature of this type of study was beyond the scope of this investigation, considering the unpredictable nature of host-pathogen interactions for the progression to infections.[198] Further work including mutagenesis and transcriptomics will be needed to validate the roles of the identified candidate loci in *S. aureus* pathogenesis. Fourth, sequences were mapped onto a reference genome, FPR3757. A limitation is that the DNA not found in the reference strain, therefore other potential loci that affect pathogenicity, was not evaluated. Further, while we used nasal carriage as our source for colonization, other sites (e.g., groin, nasopharynx, perianal areas) have also been implicated in colonization and may involve different pathogenetic mechanisms. Lastly, when predicting the clinical manifestations of infection, undoubtedly, the interplay of host factors and the environment in addition to the pathogen will need to be accounted for. We attempted to address this, in part, by merging significant clinical meta-data with genotype in the predictive model. External validation of this prediction model with larger datasets and diverse lineages are required. While an important barrier for progress in this field has been the inability to consistently prove that specific bacterial virulence determinants are responsible for clinical manifestations of infection, we have provided one step in this direction.

This study described the heterogeneity of *S. aureus* SSTIs and nasal colonization isolates. While this study was exploratory and hypothesis generating

in its approach, it identified potential novel pathogenicity mechanisms that will require further functional validation. The future challenge will be to build robust models that convert multiscale data into information that can be used to predict severity of *S. aureus* infections to rapidly tailor precision medicine in the clinic and to devise new preventative strategies.

# CHAPTER SEVEN

# Conclusions

This dissertation described the diversity of *S. aureus* population in the South Texas community, identified genetic determinants contributing to antimicrobial resistance, and characterized potential mechanisms for pathogenicity in isolates from healthy carriage and SSTIs. This chapter summarizes the major findings from the chapters, their applications, and directions for future work.

*Comparative genomics and antimicrobial resistance determinants of community-associated S. aureus in South Texas*

The evolution of community-associated *S. aureus* toward resistance to additional antibiotic classes is a major concern. This chapter demonstrated that multidrug resistance among community-associated *S. aureus* strains has emerged in South Texas. When evaluating epidemiological links, African Americans and a geographic region near a major health care setting were associated with higher proportions of multidrug-resistant strains. Moreover, the distribution of antimicrobial resistance determinants varied by clonal type. The majority of the multidrug-resistant strains were of the USA300 lineage. This study also reveals that a large proportion of the USA300 isolates sequenced are resistant to fluoroquinolone antibiotics. Further work to explore the selective pressures and role of *gyrA* mutations associated with fluoroquinolone resistance among USA300 is needed. Finally, this chapter demonstrated the high level of concordance when comparing antimicrobial resistance determinants to the phenotypically derived

antibiogram. This study is the first large dataset comprised of isolates from the primary care setting which adds to the growing literature supporting the future application of WGS in the clinical setting.

This study raises several subsequent questions regarding the drivers of antimicrobial resistance among community-associated *S. aureus* strains. A next step is to conduct a longitudinal study among infection cases and their households to reveal the patterns of transmission, the evolution of resistance during and between antimicrobial treatments, role of microbial communities, and the environment. Furthermore, transmission studies evaluating *S. aureus* at the interface of hospital and community settings might reveal novel transmission networks and evolutionary pathways of the acquisition of antimicrobial resistance.

*Genomic heterogeneity and prediction of S. aureus SSTIs*

*S. aureus* is an opportunistic pathogen that commonly exists as a colonizer but can occasionally cause infection. The most common manifestation of community associated *S. aureus* is skin and soft tissue infections. Factors revealing the pathogenetic mechanisms of why some strains go on to produce infection could result in novel effective control methods and diagnostic models. This study revealed that genomic heterogeneity exists among community associated *S. aureus* isolates causing colonization and SSTIs in South Texas. These variations may play a role in the pathogenesis of variation in pathogenicity and clinical severity. Methodologically, this study adds to the very sparse literature on using a bacterial GWAS approach. Together, this holds great promise for microbial functional genomics where experiments can be systematically designed

to test the phenotypes followed by large-scale sequencing to draw important phenotype-genotype associations.

Future work will expand on these findings toward studying the interaction between host and pathogen, linking genomic variation and their functional roles to pharmacodynamic responses, and furthering the development of using multiscale data to predict severity of infection among patients presenting with *S. aureus* infections. The application of WGS as a phenotypic prediction tool holds great potential to transform the way we manage and prevent *S. aureus* infections. In the clinical setting, WGS may be useful for deciding the course of *S. aureus* treatment at an early stage of disease. For example, a clinician may treat a highly toxic or resistant infection more aggressively with consideration for antimicrobials known to reduce toxin expression of *S. aureus* (e.g., clindamycin, linezolid). On the other hand, WGS can serve as a key tool for antimicrobial stewardship by preventing the use of broader-spectrum antimicrobials in patients with low toxicity and/or susceptible infections. Furthermore, understanding the pathogenetic mechanisms can lead to vaccine development and other important preventative strategies.

# APPENDICES

## APPENDIX A: Clinical information card

**Community-Acquired *S. aureus* in Medical Clinics: A STARNet Study**
(*Card to be completed by clinic employees who have IRB training on file with Chris Frei*)

Age:_____

Race:
- O Black
- O White
- O Other

Ethnicity:
- O Hispanic
- O Non-Hispanic

Height (inches) _____

Weight (lbs)_____

Gender:
- O Male
- O Female

History:
- O Diabetes
- O Peripheral vascular disease
- O Chronic non-infectious skin disorder
- O HIV/AIDS
- O Cancer
- O Actively receiving chemotherapy
- O Provides healthcare to others
- O Antibiotic use in last 90 days
- O History of skin infection
  - o Last 90 days?

**Study Criteria**
(*Patients who meet study criteria should be provided with an informed consent sheet. Give them at least 10 minutes to read the sheet and ask questions before obtaining their verbal consent to participate in this study. The sheet is theirs to keep.*)

Inclusion
1. Age 18 years or older
2. Without skin infection

Exclusion
1. Pregnant
2. Decisionally impaired
3. Prisoners

## APPENDIX B: *S. aureus* isolate information

| ID | Avg Covg | MLST | Zip | Case Control | Phenotype | MDR | Age | Gender | Black Race | Hispanic | Diabetes | Health-care Provider | Hx of SSTI | Hx of Abx | Hx of MRSA | BMI≥30 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A1 | 10 | 8 | 78229 | case | MRSA | 1 | 55 | Male | Yes | No | No | No | No | Yes | No | Yes |
| A4 | 9 | 8 | 78229 | case | MRSA | 0 | 31 | Male | No | No | No | No | No | No | No | No |
| A5 | 10 | 8 | 78229 | case | MRSA | 1 | 58 | Female | No | Yes | No | No | Yes | No | No | Yes |
| A8 | 10 | 8 | 78229 | case | MSSA | 0 | 27 | Male | No | Yes | No | No | No | No | No | No |
| A10 | 21 | 8 | 78229 | case | MRSA | 0 | 40 | Female | No | No | No | No | No | No | No | Yes |
| A14 | 25 | 8 | 78229 | case | MSSA | 0 | 43 | Female | No | Yes | Yes | No | No | No | No | No |
| A16 | 16 | 8 | 78229 | case | MRSA | 1 | 49 | Male | No | Yes | No | Yes | No | No | No | Yes |
| A18 | 19 | 8 | 78229 | case | MRSA | 1 | 37 | Female | No | Yes | No | No | No | No | No | Yes |
| A19 | 14 | 8 | 78229 | case | MRSA | 0 | 34 | Female | No | Yes | Yes | No | No | No | No | Yes |
| A21 | 8 | 8 | 78229 | case | MRSA | 1 | 30 | Female | No | Yes | Yes | No | Yes | Yes | No | Yes |
| A22 | 9 | 8 | 78229 | case | MRSA | 1 | 27 | Female | No | Yes | No | No | No | No | No | Yes |
| A30 | 10 | 12 | 78229 | case | MSSA | 0 | 45 | Male | No | Yes | No | No | No | No | No | No |
| A32 | 10 | 8 | 78229 | case | MRSA | 1 |  | Male | No | Yes | No | No | No | No | No | Yes |
| A34 | 9 | 8 | 78229 | case | MRSA | 0 | 54 | Male | No | No | No | No | No | No | No | No |
| A35 | 7 | 15 | 78229 | case | MSSA | 0 | 42 | Male | No | No | No | No | No | No | No | No |
| A36 | 9 | 8 | 78229 | case | MRSA | 1 | 46 | Male | No | Yes | Yes | No | No | No | No | Yes |
| B2 | 9 | unknown | 78207 | case | MRSA | 0 | 52 | Female | No | Yes | Yes | No | Yes | Yes | No | Yes |
| B4 | 10 | 8 | 78207 | case | MSSA | 0 | 19 | Male | No | Yes | No | No | No | No | No | Yes |
| B8 | 10 | 8 | 78207 | case | MSSA | 0 | 38 | Male | No | Yes | No | No | No | No | No | No |
| B9 | 12 | 8 | 78207 | case | MSSA | 0 | 44 | Female | No | Yes | No | No | Yes | No | No | No |
| B10 | 10 | 5 | 78207 | case | MSSA | 0 | 24 | Female | No | Yes | Yes | No | Yes | Yes | No | No |
| B11 | 9 | 8 | 78207 | case | MRSA | 1 | 48 | Female | No | Yes | Yes | No | No | No | No | Yes |
| B12 | 8 | 8 | 78207 | case | MRSA | 0 | 53 | Male | No | Yes | No | No | No | No | No | Yes |
| B13 | 21 | 59 | 78207 | case | MSSA | 0 | 24 | Male | No | Yes | No | No | No | No | No | No |
| B14 | 19 | 8 | 78207 | case | MRSA | 0 | 32 | Female | No | Yes | No | No | No | No | No | No |
| B15 | 18 | 8 | 78207 | case | MSSA | 0 | 41 | Male | No | Yes | No | No | No | No | No | Yes |
| B16 | 27 | 5 | 78207 | case | MSSA | 0 | 63 | Male | No | Yes | Yes | No | Yes | No | No | No |
| B18 | 18 | 8 | 78207 | case | MRSA | 0 | 31 | Male | No | Yes | No | No | No | No | No | No |
| B19 | 18 | 8 | 78207 | case | MRSA | 0 | 20 | Male | No | Yes | No | No | Yes | No | No | No |
| B20 | 18 | 8 | 78207 | case | MSSA | 0 | 66 | Female | No | Yes | Yes | No | No | No | No | Yes |
| B22 | 20 | 8 | 78207 | case | MRSA | 1 | 47 | Female | Yes | No | Yes | No | Yes | No | No | Yes |
| B23 | 19 | 8 | 78207 | case | MRSA | 1 | 23 | Female | No | Yes | No | No | No | No | No | Yes |

| | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| B25 | 17 | unknown | 78207 | case | MRSA | 0 | 29 | Female | No | No | No | No | No | Yes | No | Yes |
| B26 | 16 | 8 | 78207 | case | MRSA | 0 | 63 | Female | No | Yes | No | No | No | No | No | Yes |
| B28 | 18 | 8 | 78207 | case | MRSA | 1 | 58 | Male | Yes | No | No | No | No | No | No | No |
| B29 | 16 | 8 | 78207 | case | MRSA | 1 | 31 | Male | No | Yes | Yes | No | Yes | No | No | No |
| B30 | 22 | 8 | 78207 | case | MRSA | 0 | 30 | Male | No | Yes | Yes | No | No | No | No | Yes |
| B31 | 25 | 8 | 78207 | case | MRSA | 1 | 55 | Female | No | Yes | No | No | No | No | No | Yes |
| B32 | 23 | 8 | 78207 | case | MRSA | 1 | 25 | Male | No | Yes | No | No | No | Yes | No | No |
| B33 | 12 | 8 | 78207 | case | MSSA | 0 | 30 | Female | No | Yes | No | No | No | No | No | No |
| B35 | 10 | 8 | 78207 | case | MRSA | 1 | 26 | Female | No | Yes | No | No | No | No | No | Yes |
| B36 | 12 | 8 | 78207 | case | MRSA | 0 | 28 | Male | No | Yes | No | No | No | No | No | Yes |
| B37 | 19 | 8 | 78207 | case | MRSA | 1 | 60 | Female | No | Yes | No | No | No | No | No | Yes |
| B38 | 3 | unknown | 78207 | case | MRSA | 1 | 37 | Male | Yes | No | No | No | No | No | No | No |
| B40 | 12 | 8 | 78207 | case | MRSA | 1 | 40 | Male | No | Yes | No | No | Yes | No | No | No |
| B42 | 9 | 8 | 78207 | case | MSSA | 1 | 43 | Male | No | No | No | No | No | No | No | No |
| B45 | 10 | 8 | 78207 | case | MSSA | 1 | 49 | Male | No | Yes | No | No | No | No | No | No |
| B49 | 15 | 8 | 78207 | case | MRSA | 1 | 63 | Female | No | Yes | Yes | No | No | No | No | Yes |
| B60 | 16 | 45 | 78207 | case | MSSA | 0 | 49 | Female | No | Yes | No | No | No | No | No | Yes |
| B61 | 9 | 8 | 78207 | case | MSSA | 0 | 53 | Female | No | Yes | Yes | No | Yes | No | No | Yes |
| B62 | 12 | 8 | 78207 | case | MRSA | 0 | 49 | Female | No | Yes | No | No | No | No | No | No |
| B64 | 12 | 5 | 78207 | case | MSSA | 0 | 52 | Female | No | Yes | Yes | No | No | No | No | No |
| B65 | 12 | 8 | 78207 | case | MRSA | 0 | 19 | Female | No | Yes | No | No | No | No | No | |
| B68 | 11 | 8 | 78207 | case | MSSA | 0 | 41 | Female | Yes | No | No | No | No | No | No | Yes |
| B69 | 8 | 8 | 78207 | case | MSSA | 0 | 49 | Male | No | Yes | Yes | No | No | No | No | |
| B70 | 7 | 8 | 78207 | case | MRSA | 0 | 43 | Male | No | Yes | No | No | No | No | No | No |
| B72 | 10 | 121 | 78207 | case | MSSA | 1 | | Female | No | Yes | No | No | No | No | No | No |
| B75 | 9 | 8 | 78207 | case | MSSA | 0 | | Female | No | Yes | Yes | No | No | No | No | No |
| C1 | 11 | 8 | 78163 | case | MSSA | 0 | 65 | Male | No | No | No | No | No | Yes | No | No |
| C2 | 9 | 8 | 78163 | case | MSSA | 1 | 61 | Male | No | No | No | No | No | No | No | No |
| C3 | 9 | 8 | 78163 | case | MRSA | 0 | 24 | Male | No | No | Yes | No | No | Yes | No | No |
| C4 | 10 | 8 | 78163 | case | MRSA | 1 | 64 | Female | No | No | No | No | Yes | No | No | Yes |
| C6 | 11 | 5 | 78163 | case | MSSA | 0 | 39 | Male | No | No | No | No | No | No | No | Yes |
| C11 | 9 | 8 | 78163 | case | MRSA | 0 | 46 | Female | No | No | No | No | No | No | No | Yes |
| C19 | 13 | 8 | 78163 | case | MRSA | 0 | 12 | Male | No | No | No | No | No | No | No | No |
| C22 | 12 | 8 | 78163 | case | MRSA | 0 | 62 | Female | No | No | No | No | Yes | No | Yes | Yes |
| C35 | 13 | 8 | 78163 | case | MRSA | 1 | 49 | Male | No | No | No | No | No | No | No | No |

| | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| D1 | 10 | 8 | 78212 | case | MRSA | 0 | | Female | No | Yes | No | No | Yes | No | Yes | No |
| D7 | 8 | unknown | 78212 | case | MRSA | 1 | | Female | No | Yes | Yes | No | Yes | No | Yes | Yes |
| E3 | 10 | 8 | 78214 | case | MRSA | 1 | | Female | No | Yes | Yes | No | Yes | No | No | Yes |
| F4 | 14 | 8 | 78756 | case | MSSA | 0 | 50 | Female | No | No | No | No | No | No | No | No |
| F6 | 12 | 8 | 78756 | case | MSSA | 0 | 22 | Male | No | No | No | No | Yes | No | No | Yes |
| G3 | 9 | 59 | 78251 | case | MSSA | 0 | | Male | No | Yes | No | No | Yes | Yes | No | Yes |
| H6 | 12 | 8 | 78223 | case | MSSA | 0 | | Male | No | Yes | No | No | No | No | No | No |
| I2 | 9 | 8 | 78207 | case | MRSA | 1 | 50 | Female | No | Yes | Yes | No | No | Yes | No | |
| I3 | 10 | 8 | 78207 | case | MSSA | 0 | 37 | Female | No | Yes | Yes | No | No | No | No | No |
| 14 | 10 | 8 | 78207 | case | MSSA | | 18 | Male | No | Yes | No | No | Yes | No | No | Yes |
| I5 | 9 | 8 | 78207 | case | MRSA | 0 | 51 | Male | No | Yes | No | No | No | No | No | No |
| I7 | 10 | 8 | 78207 | case | MSSA | 0 | 25 | Male | No | Yes | No | No | No | No | No | No |
| I11 | 10 | 8 | 78207 | case | MRSA | 0 | 42 | Male | No | Yes | No | No | Yes | No | No | Yes |
| I12 | 11 | 8 | 78207 | case | MRSA | 1 | 46 | Male | No | Yes | Yes | No | Yes | No | No | Yes |
| I13 | 12 | 8 | 78207 | case | MRSA | 1 | 20 | Male | No | Yes | No | No | Yes | No | No | Yes |
| I15 | 10 | 8 | 78207 | case | MRSA | 0 | 43 | Female | No | Yes | Yes | No | No | No | No | Yes |
| I16 | 22 | 8 | 78207 | case | MSSA | 0 | 70 | Female | No | Yes | No | No | Yes | No | No | |
| I17 | 11 | 8 | 78207 | case | MRSA | 0 | 24 | Female | No | Yes | No | No | No | No | No | No |
| I21 | 10 | 8 | 78207 | case | MRSA | 0 | 31 | Male | No | Yes | No | No | No | No | No | |
| I23 | 10 | 8 | 78207 | case | MSSA | 0 | 48 | Female | No | Yes | No | No | No | No | No | Yes |
| I24 | 9 | 8 | 78207 | case | MSSA | 0 | 35 | Female | No | Yes | No | No | No | No | No | Yes |
| I27 | 10 | 8 | 78207 | case | MRSA | 1 | 37 | Male | No | Yes | No | No | No | No | No | No |
| I29 | 11 | 8 | 78207 | case | MRSA | 1 | 50 | Male | No | Yes | No | No | No | No | No | No |
| I39 | 10 | 8 | 78207 | case | MRSA | 1 | 44 | Female | No | Yes | No | No | No | No | No | Yes |
| I42 | 12 | unknown | 78207 | case | MRSA | 0 | 23 | Male | No | Yes | No | No | No | No | No | Yes |
| I54 | 11 | 8 | 78207 | case | MSSA | 0 | | Male | No | Yes | No | No | No | No | No | Yes |
| I62 | 12 | 45 | 78207 | case | MSSA | 0 | | Male | No | Yes | Yes | No | No | No | No | No |
| J4 | 13 | 188 | 78214 | case | MSSA | 0 | | Male | No | Yes | No | No | No | No | No | |
| J7 | 6 | 8 | 78214 | case | MRSA | 0 | 39 | Female | No | Yes | Yes | No | No | No | No | Yes |
| J8 | 18 | 8 | 78214 | case | MRSA | 1 | 38 | Male | No | Yes | No | No | No | No | No | |
| J11 | 15 | 15 | 78214 | case | MSSA | 0 | 57 | Female | No | Yes | Yes | No | No | Yes | No | Yes |
| J12 | 15 | 8 | 78214 | case | MRSA | 1 | 38 | Male | No | Yes | No | No | No | No | No | Yes |
| J16 | 9 | 8 | 78214 | case | MRSA | 1 | 28 | Female | Yes | No | No | No | Yes | No | No | No |
| J17 | 10 | 8 | 78214 | case | MRSA | 0 | 48 | Female | No | No | No | No | Yes | No | No | No |
| J18 | 9 | 8 | 78214 | case | MRSA | 0 | 47 | Female | No | Yes | Yes | No | Yes | Yes | No | Yes |

| | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| J19 | 10 | 8 | 78214 | case | MRSA | 1 | 34 | Female | No | Yes | Yes | No | No | No | No | Yes |
| J20 | 8.8 | 8 | 78214 | case | MRSA | 1 | 50 | Female | No | Yes | Yes | No | No | No | No | Yes |
| J26 | 19 | 8 | 78214 | case | MSSA | 0 | | Male | No | Yes | No | No | No | No | No | No |
| J28 | 18 | 8 | 78214 | case | MSSA | 0 | | Female | No | Yes | No | No | No | Yes | No | Yes |
| K2 | 12 | unknown | 78258 | case | MRSA | 0 | 53 | Female | No | No | No | No | No | No | No | |
| M2 | 8 | 8 | 78230 | case | MRSA | 1 | 38 | Male | No | Yes | No | No | Yes | No | No | Yes |
| NN2 | 11 | 8 | 78624 | case | MSSA | 0 | | Male | No | Yes | Yes | No | No | No | No | Yes |
| NN4 | 10 | 8 | 78624 | case | MSSA | 0 | | Female | No | No | No | No | No | No | No | No |
| NN6 | 10 | 8 | 78624 | case | MSSA | 1 | | Male | No | Yes | No | No | No | No | No | |
| Q2 | 11 | 8 | 78624 | case | MSSA | 0 | | Female | No | No | No | No | Yes | Yes | No | Yes |
| J1015 | 6 | 8 | 78214 | Control | MRSA | 0 | 20 | Female | No | Yes | No | No | No | No | No | Yes |
| J1019 | 18 | 45 | 78214 | Control | MSSA | 0 | 20 | Female | No | Yes | No | Yes | No | No | No | No |
| J1026 | 20 | 30 | 78214 | Control | MSSA | 0 | 48 | Male | No | Yes | Yes | No | No | No | No | No |
| J1027 | 20 | 8 | 78214 | Control | MSSA | 0 | 46 | Female | No | Yes | Yes | No | No | No | No | Yes |
| J1032 | 11 | 97 | 78214 | Control | MSSA | 0 | 39 | Female | No | Yes | No | Yes | Yes | Yes | No | Yes |
| J1036 | 18 | 188 | 78214 | Control | MSSA | 0 | 48 | Female | No | Yes | No | No | No | No | No | Yes |
| J1038 | 20 | 15 | 78214 | Control | MSSA | 0 | 50 | Male | No | Yes | No | No | No | No | No | Yes |
| J1039 | 16 | 5 | 78214 | Control | MSSA | 0 | 57 | Male | No | Yes | Yes | No | No | No | No | Yes |
| J1041 | 24 | 188 | 78214 | Control | MSSA | 0 | 60 | Female | No | Yes | No | No | No | No | No | Yes |
| J1043 | 19 | 1159 | 78214 | Control | MSSA | 0 | 18 | Male | No | Yes | No | No | Yes | Yes | No | No |
| J1050 | 21 | 45 | 78214 | Control | MSSA | 0 | 20 | Female | No | Yes | No | Yes | Yes | No | No | No |
| J1058 | 29 | 8 | 78214 | Control | MRSA | 1 | 33 | Female | No | Yes | No | No | No | No | No | Yes |
| J1060 | 25 | 6 | 78214 | Control | MSSA | 0 | 51 | Male | Yes | No | No | No | No | No | No | No |
| J1062 | 20 | 30 | 78214 | Control | MSSA | 1 | 50 | Female | No | Yes | Yes | No | No | No | No | Yes |
| J1063 | 24 | 5 | 78214 | Control | MSSA | 0 | 32 | Female | No | Yes | No | No | No | No | No | Yes |
| A1011 | 25 | 45 | 78207 | Control | MSSA | 0 | 54 | Female | No | Yes | Yes | No | No | No | No | No |
| A1015 | 19 | 5 | 78207 | Control | MSSA | 0 | 58 | Male | No | Yes | Yes | No | No | Yes | No | No |
| A1016 | 21 | 30 | 78207 | Control | MSSA | 0 | 36 | Male | No | No | No | No | No | No | No | No |
| A1017 | 20 | 30 | 78207 | Control | MSSA | 0 | 45 | Male | No | Yes | No | No | No | No | No | No |
| A1019 | 21 | 8 | 78207 | Control | MRSA | 1 | 28 | Male | Yes | No | No | No | No | No | No | No |
| A1021 | 18 | 5 | 78207 | Control | MSSA | 0 | 23 | Male | No | No | No | No | No | Yes | No | Yes |
| A1023 | 14 | 45 | 78207 | Control | MSSA | 0 | 53 | Male | No | Yes | No | No | No | No | No | No |
| A1028 | 34 | 25 | 78207 | Control | MSSA | 0 | 59 | Male | No | Yes | No | No | No | No | No | Yes |
| A1034 | 18 | 5 | 78207 | Control | MSSA | 0 | 47 | Female | No | Yes | Yes | Yes | No | No | No | Yes |
| A1053 | 16 | 5 | 78207 | Control | MSSA | 0 | 43 | Male | No | Yes | No | No | No | No | No | No |

| | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A1058 | 15 | 5 | 78207 | Control | MSSA | 0 | 48 | Male | No | Yes | No | No | No | No | No | Yes |
| A1060 | 20 | 30 | 78207 | Control | MSSA | 0 | 50 | Male | No | Yes | No | No | No | No | No | Yes |
| A1063 | 22 | 45 | 78207 | Control | MSSA | 0 | 41 | Female | No | Yes | No | No | No | Yes | No | Yes |
| A1065 | 18 | 30 | 78207 | Control | MSSA | 0 | 43 | Male | No | Yes | Yes | No | No | No | No | Yes |
| A1068 | 20 | 8 | 78207 | Control | MRSA | 1 | 42 | Male | No | Yes | Yes | No | No | No | No | No |
| B1036 | 22 | 30 | 78229 | Control | MSSA | 0 | 44 | Female | No | Yes | Yes | No | No | No | No | Yes |
| B1043 | 19 | 30 | 78229 | Control | MSSA | 0 | 26 | Female | No | No | No | No | No | No | No | No |

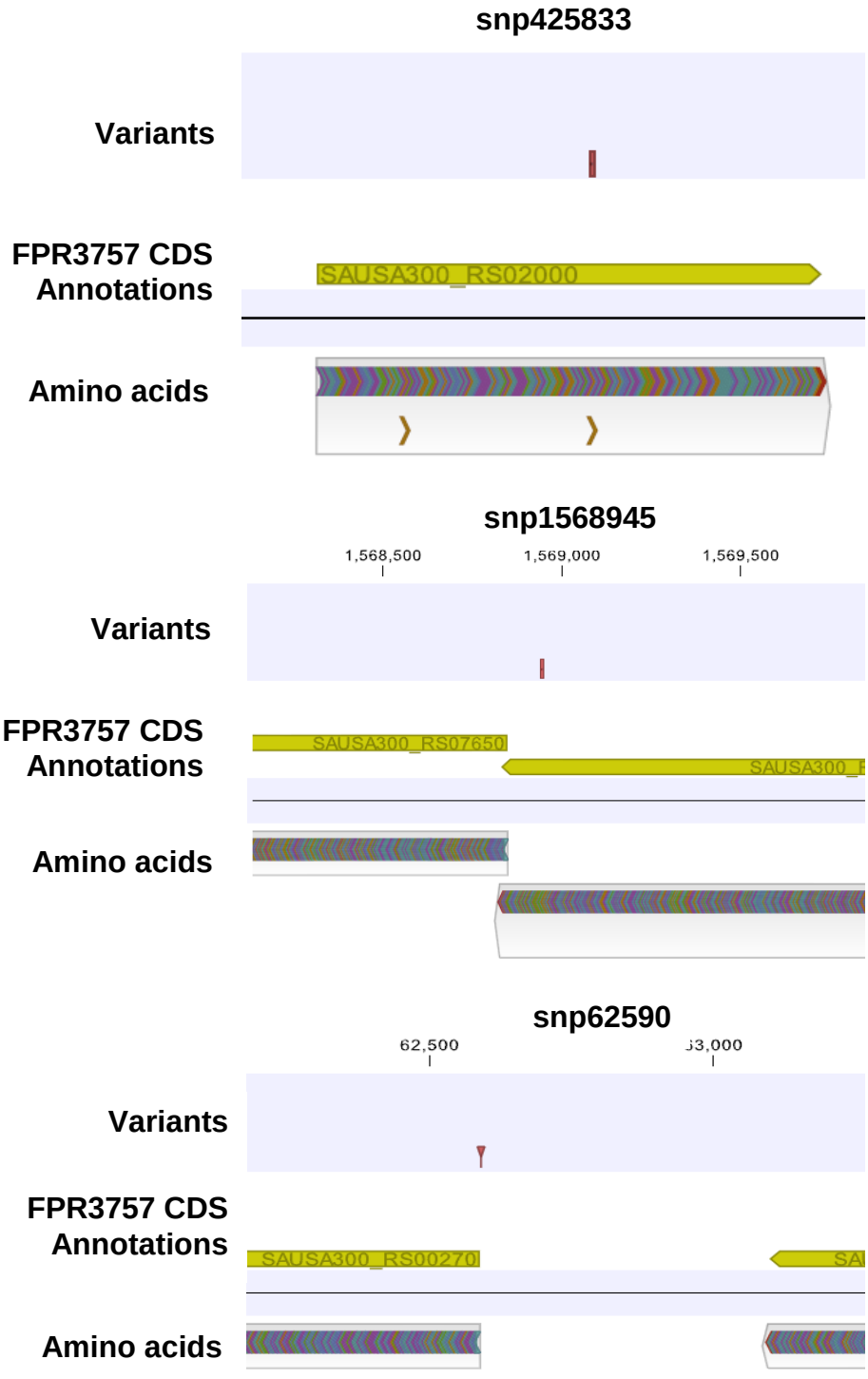
## APPENDIX C: *S. aureus* plasmid content

| | pC221 | pSJH901 | pSJH101 | pSA5 | EDINA | MSSA476 | pKH14 | pMC524/M | pMW2 | pSK3 | pSK6 | pTW20 | pWBG745 | pWBG752 | pWBG760 | Saa6159 | SAP071A | SAP101A | AP071A | pLW043 | pWBG759 | pE5 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A1 | | | 1 | 1 | | | 1 | | | | | | | | | | | | | | | |
| A1011 | | | | | 1 | | | | | 1 | | | | | | | | | | 1 | | |
| A1015 | | | | | | | | | | | | | 1 | | | | | 1 | | | | |
| A1016 | | | | 1 | | | | | | 1 | | | | | | | | | | | | |
| A1017 | | | | 1 | | | | | | 1 | | | | | | | | | | | | |
| A1019 | | | 1 | 1 | | | 1 | 1 | | | | | | | | | | | | | | |
| A1021 | | | | | | | | | | | | | | | | | | | | | | |
| A1023 | | | | | 1 | | | | | 1 | | | | | | | | | | | | |
| A1028 | | | | | | | 1 | 1 | | | | | | | | | | | | | | |
| A1034 | | 1 | | | | | | | | | | | 1 | | | | | 1 | | | | |
| A1058 | | | | | | | | | | | | | 2 | | | | | | | | | |
| A1060 | | | | 1 | | | | | | 1 | | | | | | | | | | | | |
| A1063 | | | | | | | | | | | | | | | | | | | | | | |
| A1065 | | | | 1 | | | | | | 1 | | | | | | | | | | | | |
| A1068 | | | 1 | 1 | | | 1 | | | | | | | | | | | | | | | |
| A14 | | | | 1 | | | 1 | | | | | | | | 1 | | | | | | | |
| A16 | | | | 1 | | | 1 | 1 | | | | | | | | | | | | | | |
| A18 | | | 1 | 1 | | | 1 | 1 | | | | | | | | | | | | | | |
| A19 | | | | | | | 1 | | | | | | | | | | | | | 1 | | |
| A21 | | | | | | | | | | | | | | | | | | | | | | |
| A22 | | | | 1 | | | 1 | 1 | | | | | | | | | | | | | | |
| A30 | | | | | | | | | | | | | | | | | 1 | | 1 | | | |
| A32 | | | 1 | 1 | | | 1 | | | | | | | | | | | 1 | | | | 1 |
| A34 | | | | 1 | | | 1 | | | | | | | | | | | | | | | |
| A35 | | | | | | | | | | 1 | | | | | | 1 | | | | | | |
| A36 | | | 1 | 1 | | | 1 | | | | | | | 1 | | | | | | | | |
| A4 | | | 1 | 1 | | | 1 | 1 | | | | | | | | | | | | | | |
| A5 | | | 1 | 1 | | | 1 | 1 | | | | | | | | | | | | | | |
| A8 | | | 1 | 1 | | | 1 | | | | | | | | | | | | | | | 1 |
| B10 | | | | 1 | | 1 | | | | | | | | | | | | | | | | |
| B1036 | | | | 1 | | | | | | | | | | | | | | | | | | |
| B1043 | | | | 1 | | | | | | 1 | | | | | | | | | | | | |
| B11 | | | 1 | 1 | | | 1 | 1 | | | | | | | | | | | | | | 1 |
| B12 | | | 1 | 1 | | | 1 | 1 | | | | 1 | | | | | | | | | | |
| B13 | | | 1 | 1 | | | | | | 1 | | | | | | | | | | | | |
| B14 | | | 1 | 1 | | | 1 | 1 | | | | | | | | | | | | | | |
| B15 | | | 1 | 1 | | | 1 | 1 | | | | | | | | | | | | | | |
| B16 | | | | | | | | | | | | | 2 | | | | | | | | | |
| B18 | | | 1 | 1 | | | 1 | 1 | | | | | | | | | | | | | | |
| B19 | | | 1 | 1 | | | 1 | 1 | | | | | | | | | | | | | | |
| B2 | | | 1 | 1 | | | 1 | 1 | | | | | | | | | | | | | | |
| B20 | | | 1 | 1 | | | 1 | 1 | | | | | | | | | | | | | | |
| B22 | | | 1 | 1 | | | 1 | 1 | | | | | | | | | | | | | | |
| B23 | | | 1 | 1 | | | 1 | 1 | | | | | | | | | | | | | | |
| B25 | | | | | | | | | | | | | | | | | 1 | | 1 | | | |
| B26 | | | 1 | 1 | | | 1 | 1 | | | | | | | | | | | | | | |
| B28 | | | 1 | 1 | | | 1 | 1 | | | | | | | | | | | | | | |
| B29 | | | 1 | 1 | | | 1 | 1 | | | | | | | | | | | | | | |
| B30 | | | | 1 | | | 1 | | | | | | | | | | | | | | | |
| B31 | | | 1 | 1 | | | 1 | | | | | | | | | | | 1 | | | | |
| B32 | | | 1 | 1 | | | 1 | 1 | | | | | | | | | | | | | | |
| B33 | | | 1 | 1 | | | 1 | 1 | | | | | | | | | | | | | | |
| B35 | | | 1 | 1 | | | 1 | 1 | | | | | | | | | | | | | | |
| B36 | | | 2 | 2 | | | 2 | | | | | | | | | | | | | | | |
| B37 | | | 1 | 1 | | | 1 | | | | | | | | | | | | | | | |
| B38 | | | 1 | 1 | | | | 1 | | | | | | | | | | | | | | |
| B4 | | | | 1 | | | | | | | | | | | | | | | | | | |
| B40 | | | 1 | 1 | | | 1 | 1 | | | | | | | | | | | | | | |

| | pC221 | pSIM901 | pSIM101 | pSA3 | EDINA | MSSA476 | pKH14 | pMC524/IV | pMW2 | pSK3 | pSK6 | pTW20 | pWBG745 | pWBG752 | pWBG760 | Sae6159 | SAP071A | SAP101A | AP071A | pLW043 | pWBG759 | pE5 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| B42 | | 1 | 1 | | | 1 | 1 | | | | | | | | | | | | | | | |
| B45 | | 1 | | | | 1 | | | | | | | | | | | | | | | | 1 |
| B49 | | 1 | 1 | | | 1 | | | | | | | | | | | | | | | | |
| B60 | | 1 | 1 | | | | | | | | | | | 1 | | | | | | | | |
| B61 | | 1 | 1 | | | | 1 | | | | | | | | | | | | | | | |
| B62 | | 1 | 1 | | | 1 | | | | | | | | | | | | | | | | |
| B64 | | | | | | | | | | | | 1 | | | | | | | | | | |
| B65 | | 1 | 1 | | | 1 | | | | | | | | | | | | | | | | |
| B68 | | | | | | 1 | 1 | | | | | | | | | | | | | | | |
| B69 | | 1 | 1 | | | 1 | 1 | | | 1 | | | | | | | | | | | | |
| B70 | | | | | | 1 | 1 | | | | | | | | | | | | | | | |
| B72 | | | | | | | 1 | | | | | | | | | | | | | | | |
| B75 | | 1 | 1 | | | 1 | 1 | | | | | | | | | | | | | | | |
| B8 | | | 1 | | | 1 | 1 | | | | 1 | | | | | | | | | | | |
| B9 | | | | | | | 1 | | | | | | | | | | | | | | | |
| C1 | | | | | | 1 | | | | | | | | | | | | | | | | |
| C11 | | 1 | 1 | | | 1 | 1 | | | | | | | | | | | | | | | |
| C19 | | 1 | 1 | | | 1 | 1 | | | | | | | | | | | | | | | |
| C2 | | | 1 | | | 1 | 1 | | | | | | | | | | | | | | | |
| C22 | | 1 | 1 | | | 1 | | | | | | | | | | | | | | | | |
| C3 | | 1 | 1 | | | 1 | 1 | | | | | | | | | | | | | | | |
| C35 | | 1 | 1 | | | 1 | | | | | | | | | | | | | | | | |
| C4 | | 1 | 1 | | | 1 | | | | | | | | | | | | | | | | |
| C6 | | | | | | | 1 | | | | | 1 | | | | | | | | | | |
| D1 | | 1 | 1 | | | 1 | 1 | | | | | | | | | | | | | | | |
| D7 | | 1 | 1 | | | | 1 | | | | | | | | | | | | | | | 1 |
| E3 | | 1 | 1 | | | 1 | 1 | | | | | | 1 | | | | | | | | | 1 |
| F4 | | | | | | 1 | 1 | | | | | | | | | | | | | | | |
| F6 | | | | | | 1 | | | 1 | | | | | | | 1 | | | | | | |
| G3 | | 1 | 1 | | | | | 1 | 1 | | | | | | | | | | | | | |
| H6 | | | | | | 1 | | | | | | | | | | | | | | | | |
| I11 | | 1 | 1 | | | 1 | | | | | | | | | | | | | | | | |
| I12 | | 1 | 1 | | | 1 | 1 | | | | | | | | | | | | | | | |
| I13 | | 1 | 1 | | | 1 | 1 | | | | | | | | | | | | | | | |
| I15 | | 1 | 1 | | | 1 | 1 | | | | | | | | | | | | | | | |
| I16 | | 1 | 1 | | | 1 | 1 | | | | | | | | | | | | | | | |
| I17 | | | 1 | | | 1 | 1 | | | | | | | | | | | | | | | |
| I2 | | 1 | 1 | | | 1 | 1 | | | | | | | | | | | | | | | 1 |
| I21 | | 1 | 1 | | | 1 | | | | | | | | | | | | | | | | |
| I23 | | 1 | 1 | | | 1 | | | | | | | 1 | | | | | 1 | | | | |
| I24 | | 1 | 1 | | | 1 | 1 | | | | | | 1 | | | | | | | | | |
| I27 | | | 1 | | | 1 | 1 | | | | | | | | | | | | | 1 | 1 | |
| I29 | | 1 | 1 | | | | | | | | | | | | | | | | | | | |
| I3 | | | | 1 | | 1 | | | 1 | | | | | | | | | | | | | |
| I39 | | 1 | 1 | | | 1 | | | | | | | | | | | | | | | | |
| I4 | | | 1 | | | 1 | 1 | | | | | | | | | | | | | | | |
| I42 | | 1 | 1 | | | 1 | 1 | | | | | | | | | | | | | | | |
| I5 | | 1 | 1 | | | 1 | | | | | | | | | | | | | | | | |
| I54 | | 1 | 1 | | | 1 | | | | | | | | | | | | 1 | | | | |
| I62 | | | 1 | | | | | | | | | | | 1 | | | | | | | | |
| I7 | | | 1 | | | 1 | 1 | | | | | | | | | | | | | | | |
| J1026 | | | 1 | | | | | | 1 | | | | | | | | | | | | | |
| J1062 | | | 1 | | | | | | 1 | | | | | | | | | | | | | |
| J11 | | | | | | | | | | | | | | | | | | | | | | |
| J4 | | | | | | | | | 1 | | | | | | | 1 | | | | | | |
| J7 | | 1 | 1 | | | 1 | 1 | | | | 1 | | | | | | | | | | | |
| J8 | | 1 | 1 | | | 1 | 1 | | | | | | | | | | | | | | | |
| J12 | | 1 | 1 | | | 1 | 1 | | | | | | | | | | | | | | | |
| J16 | | 1 | 1 | | | 1 | 1 | | | | | | | | | | | | | | | |
| J17 | | 1 | 1 | | | 1 | 1 | | | | 1 | | | | | | | | | | | |
| J18 | | 1 | 1 | | | 1 | 1 | | | | | | | | | | | | | | | |

| | pC221 | pSIM901 | pSIM101 | pSAS | EDINA | MSSA476 | pKH14 | pMC524/IV/pMW2 | pSK3 | pSK6 | pTW20 | pWBG745 | pWBG752 | pWBG760 | Sae6159 | SAP071A | SAP101A | AP071A | pLW043 | pWBG759 | pE5 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| J19 | | | 1 | 1 | | | 1 | 1 | | | | | 1 | | | | | | | | |
| J20 | | | 1 | 1 | | | 1 | | | | | | | | | | 1 | | | | |
| J28 | | | | | | | 2 | 2 | | | | | | | | | | | | | |
| J1015 | | | | | | | 1 | | | | | 1 | | | | | | | | | |
| J26 | | | | | | | 1 | 1 | | | | | | | | | | | | | |

## APPENDIX D: Loci of significant variants



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